Elucidation of underlying mechanisms of pathogenesis in the entomologenous fungus *Metarhizium anisopliae*

Thesis

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ELUCIDATION OF UNDERLYING MECHANISMS OF PATHOGENESIS IN THE ENTOMOGENOUS FUNGUS *METARHIZIUM ANISOPLIAE*

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In memory of my beloved father

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Abstract

In order to determine the effects of nutrition on underlying mechanisms of attenuation of the entomogenous fungus *Metarhizium anisopliae*, a series of *in vitro* and *in vivo* studies were undertaken. Nutritional composition of culture media influenced germination, growth, conidiation, yield, and accumulation of endogenous reserves, conidial attributes and virulence. Addition of KCl to SDA media (SDAM) significantly reduced germination of the three isolates *in vitro* and lowered germination and appressorial development on the cuticles of potato-peach aphids and pollen beetles. Conidia grown on SDAM and minimal medium (MM) were more aggressive than conidia derived from SDA or Yeast Extract Agar (YEA). Results also identified that culture media influenced virulence of *M. anisopliae* isolates by influencing the fungal physiological qualities such as presence or absence of erythritol and arabitol, and the balance of carbon and nitrogen (C:N ratio) in conidia. Dark, pigmented conidia from aphid cuticles or nutrient-limited MM media bound less lectins and calcofluor, and accumulated higher concentration of polyols and proteins, had higher germination potential when placed on insect cuticles, formed appressoria more frequently and were highly efficacious against aphids and pollen beetles than conidia from nutrient-rich SDA media. The level of virulence was also affected by the ability of conidia to adhere to the cuticle of aphids and beetles. A strong linear relationship between fluorescence intensity of calcofluor treated conidia and the number of conidia adhered to the insect cuticles, where increasing spore fluorescence corresponded to increased spore adhesion, suggested an important role of ß-glucans in pathogen-host interaction mechanism.

Further studies were conducted to determine the effects of repeated sub-culturing on nutrient-poor and –rich media on morphology, phenotype, pathogenicity and physiology

of resultant inocula. Results revealed that eleven consecutive passages of the three *M. anisopliae* isolates on SDA media significantly changed colony colour, increased sectoring and production of sterile colonies, reduced sporulation and germination potential of harvested conidia. In contrast, passaging on MM eleven times in succession had no effect on the morphology or phenotype of the pathogen. Continuous sub-culturing on both SDA and MM media influenced the ability of *M. anisopliae* isolates to absorb various carbohydrates including glucose. This in turn, influenced the accumulation of reserves carbohydrates such as glycerol, mannitol and affected the expression of surface carbohydrates such as ß-glucans and surface proteins such as hydrophobins. Increased concentration of endogenous carbon, polyols, surface carbohydrates and hydrophobins in attenuated inocula were associated with increased adherence to and reduced germination on aphid cuticles. Reduced abilities of attenuated inocula to germinate faster and failure to produce appressoria resulted in increased LT_{50} values and thus reduced virulence.

There was no link established between virulence, as measured by LT_{50} values, and Pr1 production. *Pr1* gene expression was observed in both attenuated and non-attenuated sub-cultures of *M. anisopliae* isolates, suggesting that repeated sub-culturing had no effect on gene expression. Although pathogenicity of the pathogen was not affected by repeated sub-culturing on both SDA and MM media, the key attributes of virulence such as LT_{50} and LC_{50} were significantly affected, where increased time and dose taken to initiate and complete disease development corresponded with increased LT_{50} and LC_{50} values.

CONTENT

Acknowledgments		i
Abstract		ii
Content		iv
List of publications		ix
List of tables		x
List of figures		xiii
List of plates		xv
Chapter 1:	**General Introduction**	1
1.1	Biological control of insect-pests	2
1.2	*Metarhizium anisopliae* as biocontrol agent	5
1.3	Potato-peach aphid (*Myzus persicae* (Sulzer))	6
1.4	The concept of pathogenicity and virulence	11
	1.4.1 Attenuation of virulence	12
1.5	Nutrition and pathogenesis	17
	1.5.1 Endogenous reserves in fungi	18
	1.5.2 Ecophysiological manipulation of endogenous reserves in fungal BCAs	20
	1.5.3 Occupation of the exoskeleton	23
	1.5.4 Attachment	24
	1.5.5 Germination	28
	1.5.6 Cuticle penitration	31
1.6	Aims and objectives	38

Chapter 2:	**The effects of nutrition on morphological, physiological and pathogenic state of the entomopathogenic fungus, *Metarhizium anisopliae***		40
2.1	Introduction		41
2.2	Materials and methods		43
	2.2.1	Collection and rearing of *M. aeneus* and *M. persicse*	43
	2.2.2	Maintenance and storage of *M. anisopliae*	43
	2.2.3	Effect of artificial media on the germination, growth and sporulation of *M. anisopliae in vitro*	43
	2.2.4	Effect of artificial media on virulence of *M. anisopliae* against *M. persicse*	45
	2.2.5	Effect of artificial media on virulence of *M. anisopliae* against *M.aeneus*	46
	2.2.6	Effect of media on the carbohydrate residues at the conidial surface	47
	2.2.7	Polyols and trehalose analysis	49
	2.2.8	Total Carbon, Sulphur and Nitrogen analysis	50
	2.2.9	Analytical isoelectric focusing (IEF)	51
	2.2.10	SDS-Polyacrylamide gel electrophoresis	52
2.3	Results		52
	2.3.1	Effect of artificial media on the germination, growth and sporulation of *M. anisopliae in vitro*	52
	2.3.2	Effect of culture media on virulence of *M. anisopliae*	57
	2.3.3	Adhesion and germination of *M. anisopliae* conidia on insect cuticles	61
	2.3.4	Effect of media on the carbohydrate residues at the conidial surface	67
	2.3.5	Polyols and trehalose content	73
	2.3.6	Carbon, Sulphur and Nitrogen content	75
	2.3.7	Electrophoretic analysis	77

2.4	Discussion	80
Chapter 3:	**The effects of successive *in vitro* sub-culturing on morphological and physiological changes in the entomopathogenic fungus, *Metarhizium anisopliae***	89
3.1	Introduction	90
3.2	Materials and methods	91
	3.2.1 Origin and maintenance of *Metarhizium anisopliae* isolates	91
	3.2.2 Assessment of *in vitro* germination, growth and sporulation	92
	3.2.3 Electrophoretic studies	93
	3.2.4 Assessment of *in vivo* adhesion, germination, differentiation of appressoria and sporulation	94
	3.2.5 Adhesion of conidia and germlings to artificial surface	95
3.3	Results	96
	3.3.1 Germination, growth and morphological phenotype of the three *M. anisopliae* isolates *in vitro*	96
	3.3.2 Isozyme electrophoresis	102
	3.3.3 The germination behaviour of the three *M. anisopliae* isolates *in vivo*	104
	3.3.4 Mortality and mean survival times of *M. persicse* infected With *M. anisopliae*	110
	3.3.5 The adhesion and germination behaviour of the three *M. anisopliae* isolates on artificial surface	112
3.4	Discussion	125
Chapter 4:	**The effect of successive *in vitro* sub-culturing on pathogenicity of the entomopathogenic fungus, *Metarhizium anisopliae***	131
4.1	Introduction	132
4.2	Materials and methods	134
	4.2.1 Virulence tests	134

4.2.1.1 Cumulative mortalities, LC_{50} and LT_{50} studies 135

4.2.2 Isozyme analysis 135

4.2.3 Enzyme studies 136

4.2.3.1 Effect of successive sub-culturing on protease (Pr1) production 136

4.2.3.2 DNA extraction 137

4.2.3.3 Amplification of Pr1 gene 138

4.3 Results 139

4.3.1 Effect of successive sub-culturing on dose-time-mortality relationship and mycosis development of three isolates of *M. persicse* 139

4.3.2 Electrophoretic studies 151

4.3.3 Enzymatic analysis 153

4.3.3.1 Pr1 production: quantitative enzyme assay 153

4.3.3.2 *pr1* gene expression: nested PCR 155

4.4 Discussion 158

Chapter 5: The effect of successive *in vitro* sub-culturing on physiological state of *Metarhizium anisopliae* conidia and its relation to virulence 165

5.1 Introduction 166

5.2 Materials and methods 170

5.2.1 Assimilation of carbon substrates 170

5.2.2 Total Carbon, Sulphur and Nitrogen analysis 170

5.2.3 Polyols and trehalose analysis 176

5.2.4 Extraction, quantification and characterisation of conidial surface proteins 176

5.3 Results 179

5.3.1 Carbohydrate utilisation by virulent and attenuated conidia of three *M. anisopliae* isolates 179

5.3.2 Accumulation of endogenous carbon, nitrogen and sulphur in virulent and attenuated conidia of three *M. anisopliae* isolates 181

5.3.3 Accumulation of endogenous polyols and trehalose in and attenuated conidia of three *M. anisopliae* isolates 184

5.3.4 Expression of surface proteins during continued *in vitro* sub-culturing 186

5.4 Discussion 190

Chapter 6: General discussions 197

6.1 Nutrition and pathogenesis 198

6.2 Nutrition and attenuation 204

References 213

LIST OF PUBLICATIONS

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LIST OF TABLES

Table	Title	Page
1.1	Host range of the most common entomogenous fungi	4
1.2	Fungi registered and commercially marketed as biological control agents of pests (revised from Butt & Copping, 2000; Wraight *et al.*, 2001)	6
1.3	Cuticle-degrading enzymes of entomogenous fungi	13
2.1	Conidia production by three isolates of *Metarhizium anisopliae* cultured on different culture media 21 days after inoculation	59
2.2	Percentage mortality and lethal time (LT_{50}) obtained for three isolates of *Metarhizium anisopliae* cultured on different growth media against *Myzus persicae* following 7 days incubation at 23 °C	60
2.3	Percentage of germinated conidia and of germlings which had produced appressoria on the cuticle of *Myzus persicae* individuals of three isolates of *Metarhizium anisopliae* grown on different media 43 hours after inoculation	62
2.4	Percentage mortality, lethal time (LT_{50}) and conidial germination behaviour recorded for isolate V245 of *Metarhizium anisopliae* cultured on different growth media on *Meligethes aeneus* (pollen beetle)	63
2.5	Fluorescence intensity (arbitrary units) of conidia as determined by an LS 30 luminescence spectrometer operating at an excitation wavelength of 350 nm and an emission wavelength of 435 nm for three isolates of *Metarhizium anisopliae* cultured on different growth media (transformed logged base 10 data)	69
2.6	Effect of culture media on Fluorescence Index§ for the lectins Con (A), WGA and RCA bound to conidia of three isolates of Metarhizium anisoplia	72
2.7	Accumulation of endogenous carbon, nitrogen and sulphur (mg g^{-1} dry weight) in conidia of three isolates of *Metarhizium anisopliae* cultured on different media as determined by SC-144DR (Carbon/Sulfur) and FP-528 (Protein/Nitrogen) Analysers	76

2.8	Mean total protein content of crude extracts obtained from conidia of three isolates of *Metarhizium anisopliae* cultured on different culture media	**80**
2.9	Relationships between endogenous reserves accumulated in conidia of three isolates of *Metarhizium anisopliae* cultured on different media and germination, and between endogenous reserves and virulence of such conidia	**85**
3.1	Effect of *in vitro* sub-culturing on the germination of three isolates of *Metarhizium anisopliae* following 24 h incubation at 23°C on SDA	**97**
3.2	Effect of *in vitro* sub-culturing on the phenotype of three isolates of *Metarhizium anisopliae* following 15 d incubation at 23°C on SDA	**101**
3.3	Effect of *in vitro* sub-culturing on conidia production by three isolates of *Metarhizium anisopliae* 15 days after inoculation	**102**
3.4	Percentage of germinated conidia and of germlings which had produced appressoria on the cuticle of *Myzus persicae* individuals 24 h post-inoculation for three isolates of *Metarhizium anisopliae* sub-cultured on SDA and MM artificial media	**105**
3.5	Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA and MM artificial media on conidia, germtube and appressorium dimentions following incubation at 23°C for 24 h	**106**
3.6	Effect of sub-culturing three isolates of *Metarhizium anisopliae* on SDA and MM artificial media on mortality and lethal time (LT_{50}) against *Myzus persicae* following 7 days incubation at 23°C	**111**
4.1	Effect of successive *in vitro* sub-culturing on conidia production on dead *Myzus persicae* inoculated with 10^7 conidia ml^{-1} for three isolates of *Metarhizium anisopliae* conidia sub-cultured on SDA and MM media and incubated for 14 days at 23 °C	**147**
4.2	Effect of successive sub-culturing on median lethal concentration (LC_{50}) obtained for three isolates of *Metarhizium anisopliae* sub-cultured on SDA and tested against *Myzus persicae* following 7 days incubation at 23 °C	**148**
4.3	*Metarhizium anisopliae* sub-cultured on MM and tested against *Myzus persicae* following 7 days incubation at 23 °C	**149**

4.4	Percentage germination of three isolates of *Metarhizium anisopliae* conidia produced by repeated sub-culturing on SDA following 18 h incubation period in MLM amended with cockroach homogenate and SDB at 23 °C	**154**
4.5	Effect of *in vitro* sub-culturing on SDA on Pr1 activity, total protein and biomass of three isolates of *Metarhizium anisopliae* cultured in MM liquid media containing cockroach homogenate for 63 hr at 23 °C	**155**
5.1	Carbon source utilisation profiles of *M. anisopliae* isolates as determined on API 50 CH strips following successive sub-culturing on SDA media	**180**
5.2	Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA and MM media on the accumulation of total carbon, nitrogen (C:N) and sulphur in conidia	**183**
5.3	Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA on the accumulation of endogenous polyols (mg g^{-1} dry weight) in conidia	**185**
5.4	Accumulation of endogenous polyols and trehalose (mg g^{-1} dry weight) in conidia of sub-culture 1 for three isolates of *Metarhizium anisopliae* cultured on MM medium	**186**
5.5	Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA medium on the total amount of spore surface proteins extracted after sequential four treatments with SDS and one treatment with FA	**188**
5.6	Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA medium on fluorescence intensity (arbitrary units) of conidia as determined by an LS 30 luminescence spectrometer operating at an excitation wavelength of 350 nm and an emission wavelength of 435 nm	**189**

LIST OF FIGURES

Figure	Title	Page
1.1	Schematic representation of pathogen-host interaction in relation to the link between nutrition and pathogenesis	25
2.1a-c	Effect of culture media on the germination of three isolates of *Metarhizium anisopliae*; V245 (a), V208 (b) and V234 (c) following 24 h incubation at 23 °C. SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. The bars represent percentage of standard errors	54
2.2a-c	Effect of culture media on colony diameter of three isolates of *Metarhizium anisopliae*; V245 (a), V208 (b) and V234 (c) following incubation for 15 d at 23 °C. SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. The bars represent standard errors	56
2.3	Relationship between fluorescence intensity and the number of conidia attached to the cuticle of *Myzus persicae* for three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) grown on a range of culture media. •-SDA- = Sabouraud Dextrose Agar; ■-YEA- = Yeast Extract Agar and ▲-MM+ = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. Values shown in parentheses are correlation coefficient values (r)	71
2.4	Comparison of the ratios of polyols/trehalose present in conidia of *Metarhizium anisopliae* isolates V245 (a), V208 (b) and V234 (c) grown on a range of culture media and aphids	74
3.1	Effect of sub-culturing on colony diameter of three isolates of *Metarhizium anisopliae* ; V245 (●), V208 (■) and V234 (▲) following incubation for 15 d at 23° C on SDA (Sabouraud Dextrose Agar). 1,3,5,7,9,11 are successive sub-cultures. Each data point is the means of five replicate cultures	98
3.2a-d	Effect of sub-culturing on SDA on the number of V245 conidia adhering to the polystyrene surface following incubation for 24, 48 and 72 h at 23° C in: distilled water (a), 1% (w/v) glucose (b), 1% (w/v) yeast extract (c) and combination of 1% glucose and 1% yeast extract (d). LSD (Med*Sub-cult*Time) = 11.9, $P < 0.001$	113

3.3a-d	Effect of sub-culturing on SDA on germination of V245 conidia following incubation for 24, 48 and 72 h at 23° C in distilled water, 1% (w/v) glucose, 1% (w/v) yeast extract and combination of 1% glucose and 1% yeast extract. Bars are standard errors of means	**115**
4.1a-c	Effect of successive *in vitro* sub-culturing on SDA on % mortality of *Myzus persicae* caused by three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) following 7 days incubation at 23 °C at different spore concentrations (10^4-10^8 conidia ml^{-1})	**140**
4.2a-c	Effect of successive *in vitro* sub-culturing on SDA on % mycoses of *Myzus persicae* caused by three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) following 14 days incubation at 23 °C at different spore concentrations (10^4-10^8 conidia ml^{-1})	**142**
4.3a-c	Effect of successive *in vitro* sub-culturing on MM on % mortality of *Myzus persicae* caused by three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) following 7 days incubation at 23 °C at different spore concentrations (10^3-10^7conidia ml^{-1})	**143**
4.4a-c	Effect of successive *in vitro* sub-culturing on MM on % mycoses of *Myzus persicae* caused by three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) following 14 days incubation at 23 °C at different spore concentrations (10^3-10^7conidia ml^{-1})	**144**
4.5a-c	Effect of successive *in vitro* sub-culturing on SDA on LT_{50} values obtained for three isolates of *Metarhizium anisopliae* V245 (a) ($p<0.01$ at 10^6-10^8 conidia ml^{-1}), V208 (b) ($p<0.01$ at 10^5-10^7 conidia ml^{-1}) and V234 (c) ($p<0.01$ at 10^5-10^8 conidia ml^{-1}) at different spore concentrations (10^4-10^8 conidia ml^{-1}) following 7 days incubation at 23 °C	**150**
4.6a-c	Effect of successive *in vitro* sub-culturing on MM on LT_{50} values obtained for three isolates of *Metarhizium anisopliae* V245 (a) ($p<0.01$ at 10^3-10^4 conidia ml^{-1}), V208 (b) ($p<0.01$ at 10^4-10^6 conidia ml^{-1}) and V234 (c) ($p<0.05$ at 10^4-10^6 conidia ml^{-1}) at different spore concentrations (10^3-10^7 conidia ml^{-1}) following 7 days incubation at 23 °C	**152**
5.1	Analytical flow diagram of SC-144DR Carbon/Sulphur Analyser (from Manual, LECO Corporation, USA)	**173**
5.2	Analytical flow diagram of FP-528 Protein/Nitrogen Analyser (from Manual, LECO Corporation, USA)	**175**

LIST OF PLATES

Plate	Title	Page
1.1	Larvae and adults of oviparous females *Myzus persicae* (Sulzer) on fresh leaves of Chinese cabbage	7
2.1a-d	Effect of culture media on germination behaviour of *Metarhizium anisopliae*,x 400; V208 on SDAM- (a), V208 on SDAM+ (b), V234 on MM- (c) and V234 on MM+ (d) following 24 h incubation at 23 °C. SDAM = SDA amended with 55 g l^{-1} KCl, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate	55
2.2a-c	Effect of culture media on cultural and morphological characteristics of three isolates of *Metarhizium anisopliae*; V245 (a), V208 (b) and V234 (c) following incubation for 15 d at 23 °C. 1. SDA = Sabouraud Dextrose Agar, 2. SDAM = SDA amended with 55 g l^{-1} KCl, 3. YEA = Yeast Extract Agar, 4. MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate	58
2.3a-d	Germination behaviour of *Metarhizium anisopliae* isolate V245 on aphid cuticle following incubation for 43 hr at 23 °C (fluorescence microscopy, x1000). a. two germinated conidia, one of which produced a long, narrow appressorium directly from conidium (arrows) (SDA-); b. conidia harvested from SDAM- , one of the conidium produced an appressorium and a penetration peg (arrow); c. YEA-grown conidium formed an appressorium on the end of relatively short germ-tube; d. a complex appressorium formed on the end of a long germ-tube (MM media). SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate	65

2.4a-d	Scanning electron micrographs (SEM) of conidia of *Metarhizium anisopliae* isolate V245 on aphid cuticle following incubation for 43 hr at 23 °C. a. SDAM-grown conidia. Note those conidia that had germinated produced no appressoria. Bar =10 μm; b. same conida as in a. Note mucilage released along the thin germ-tube (arrows). Bar = 5 μm; c. MM-grown conidia produced large single appressoria at the end of the long germ-tubes. Bar = 10 μm; d. same as in c. Note a great amount of mucilage secreted along the germ-tube and around appressorium. Bar = 5 μm. SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate	**66**
2.5a-k	a-d. Conidia of *Metarhizium anisopliae* isolate V208 stained with calcofluor emit blue fluorescence of different intensity (x1000). a. conidia harvested from SDAM-; b. conidia harvested from SDA-; c. conidia harvested from YEA-; d. conidia harvested from MM-. e-g. Reactivity of WGA lectin with the cell walls of conidia of *Metarhizium anisopliae,* isolate V208 grown on SDAM. e. strong fluorescence; f. moderate fluorescence; g. weak fluorescence. h. reactivity of Con A lectin with the cell wall of a conidium (V208) harvested from SDAM. Note fluorescence of the material deposited to the surface of this conidium (arrow). Bars = 10 μm. i-k. SEM of conidia harvested from: i. SDAM- and k. MM-. Bar = 10 μm. SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate	**68**
2.6a-b	a. Analytical IEF (pH 4-6.5) of water-extracted proteins from conidia of three isolates of *Metarhizium anisopliae* V245, V208 and V234 harvested from SDA- (lane 1), SDA+ (lane 2), SDAM- (lane 3), SDAM+ (lane 4),YEA- (lane 5), YEA+ (lane 6), M (pI maker proteins); b. SDS-PADE of water-extracted proteins from conidia of two isolates of *Metarhizium anisopliae* V245 and V208 harvested from SDA- (lane 1), SDA+ (lane 2), SDAM- (lane 3), SDAM+ (lane 4), YEA- (lane 5), YEA+ (lane 6), MM- (lane 7), MM+ (lane 8), M (kDa marker proteins)	**78**
3.1	Successive sub-culturing of *Metarhizium anisopliae* on SDA caused isolate V208 to become sterile	**100**
3.2a-b	Comparative non-specific esterase banding patterns of isolate V245 separated by slab gel electrophoresis: a. conidia harvested from sporulating aphid cadavers (lane 1) and profusely sporulating sub-cultures: one (lane 2), three (lane	**103**

3), five (lane 4), seven (lane 5), nine (lane 6) and eleven (lane 7); b. conidia and mycelia harvested from sub-culture three: morphologically similar to sub-culture one (lane 1). Producing white mycelia (lane 2) and producing yellow mycelia (lane 3). M (marker dye). Rm (relative electrophoretic mobility)

3.3a-e Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V208 on aphid cuticle following incubation for 24 h at 23 °C (fluorescence microscopy, x1000): a. a conidium of sub-culture 1 from SDA produced a long and thick appressorium on the end of relatively short germ-tube; b. similar to that of a. conidia of sub-culture 5 from SDA formed large appressoria; c. a great number of conidia of sub-culture 9 adhered to the aphid cuticles; d. conidia of sub-culture 1 passaged through MM produced appressoria on the end of a long germ-tube; e. conidium of sub-culture 9 passaged through MM produced significantly longer and thinner germtubes than that in d. SDA = Sabouraud Dextrose Agar, MM = Minimal Medium **108**

3.4a-f Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V234 on aphid cuticle following incubation for 24 h at 23 °C (fluorescence microscopy, x1000): a. conidia of sub-culture 1 passaged through SDA; b. greater number of attenuated conidia (sub-culture 3 from SDA) attached to aphid cuticle in comparison to sub-culture 1, however, very few germinated and produced appressoria; c. similar to sub-culture 3 germination of attenuated sub-culture 5 is slow with smaller number of germlings producing appressoria; d. a large number of conidia of sub-culture 11 passaged through SDA produced large appressoria; e. conidia of sub-culture 9 passaged through MM produced considerably longer germtubes than that in c. and formed small appressoria on the end of a long germetube (note penetration pegs (arrows)); f. more aggressive conidium produced significantly larger appressorium than that of less aggressive conidia in e. SDA = Sabouraud Dextrose Agar, MM = Minimal Medium **109**

3.5a-f Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 24 h at 23 °C in distilled water (fluorescence microscopy, x1000): a. sub-culture 1; b. sub-culture 3; c. sub-culture 5; d. sub-culture 7; e. sub-culture 9; f. sub-culture 11. Note that although there was no germination observed in any of the tested sub-cultures, the was a clear difference in the intensity of calcoflour fluorescence **116**

3.6a-f	Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 48 h at 23 °C in distilled water (fluorescence microscopy, x1000): a. sub-culture 1; b. sub-culture 3; c. sub-culture 5; d. sub-culture 7; e. sub-culture 9; f. sub-culture11. Note that although germination of conidia occurred in all sub-cultures, the most advanced mycelial development was recorded for sub-culture 3	**117**
3.7a-d	Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation at 23 °C in glucose (fluorescence microscopy, x1000): a. sub-culture 9 for 24 h; b. sub-culture 3 for 48 h; c. sub-culture 5 for 48 h; d. sub-culture 9 for 48 h. Note a spontaneous mycelial development in sub-culture 5	**118**
3.8a-c	Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 72 h at 23 °C in glucose (fluorescence microscopy, x1000): a. sub-culture 3; b. sub-culture 5 c. sub-culture 11. Note that only sub-culture 3 produced a large number of submerged conidia, whereas sub-culture 5 formed a great number of appressoria of different shape and size. In contrast, a poorly developed germtube was typical to sub-culture 11	**119**
3.9a-d	Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 24 h at 23 °C in yeast extract (fluorescence microscopy, x1000): a. sub-culture 3; b. sub-culture 7; c. sub-culture 9; d. sub-culture11. Note advanced germination and mycelial branching was characteristic to sub-culture 3 only	**120**
3.10a-h	Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 48 h (a-d) and 72 h (e-h) at 23 °C in yeast extract (fluorescence microscopy, x1000): a. sub-culture 5; b. sub-culture 7; c. sub-culture 9; d. sub-culture11; e. sub-culture 1; f. sub-culture 3; g. sub-culture 5; h. sub-culture 9. Note that yeast extract induced appressorial formation after 48 h incubation for sub-culture 5 only. Distinctive dipolar germination behaviour was recoded for sub-cultures 7, 7 and 11. Following 72 h incubation period a large number of simple and complex appressoria were developed in sub-cultures 3, 5 and 9	**122**
3.11a-g	Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 24 h (a-d) and 48 h (e-g) at 23 °C in	**123**

yeast extract and glucose (fluorescence microscopy, x1000): a. sub-culture 3; b. sub-culture 5; c. sub-culture 7; d. sub-culture 9; e. sub-culture 1; f. sub-culture 7; g. sub-culture 9. Note that addition of glucose to yeast extract activated spore germination for almost all sub-cultures tested suppressing extensive hyphal growth in sub-culture 7 and stimulating the growth in sub-culture 9 after 24 h incubation. However, after 48 h incubation period a spontaneous elongation of hyphae was observed in sub-culture 7 only

3.12a-c Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 72 h at 23 °C in yeast extract and glucose (fluorescence microscopy, x1000): a. sub-culture 3; b. sub-culture 7; c. sub-culture 9. Note extensive hyphal development for sub-cultures 3, 7 and 9. Only sub-culture seven produced large, often branching, appressoria **124**

4.1a-b External sporulation of V245 isolate on aphid cadavers treated with conidial suspension: a. at 10^4 conidia ml^{-1} and b. at 10^7 conidia ml^{-1} following 14 days incubation at 23 °C **145**

4.2a-c Analytical IEF (pH 4-6.5) of water-extracted proteins from conidia of three isolates of *Metarhizium anisoplia:* V245 (a), V208 (b) and V234 (c) harvested from sub-cultures: 1 (lane 1), 2 (lane 2), 3 (lane 3), 4 (lane 4), 5 (lane 5), 6 (lane 6), 7 (lane 7), 8 (lane 8), 9 (lane 9) and 10 (lane 10). M (pI marker proteins) **157**

4.3 Detection of prl PCR product of *Metarhizium anisoplia* isolate V 208 DNA extracts from mycelia in Saburaud Dextrose Broth of sub-cultures: 1 (lane 1), 3 (lane 2), 5 (lane 3), 7 (lane 4), 9 (lane 5) and 11 (lane 6) **157**

5.1a-b SDS-PAGE protein profile of conidia of isolates V245 (a) and V208 (b) sub-cultured once on SDA media and treated with hot SDS once (lanel), twice (lane 2), three times (lane 3), four times (lane4) and then treated with formic acid (lane 5) and subsequently subjected to performic acid treatment (lane 6). M (protein markers) **187**

5.2a-b SDS-PAGE of rodlet proteins dissolved in formic acid (a) from conidia harvested from sub-cultures 9 (lane 1), 7 (lane 2), 5 (lane 3), 3 (lane 4) of isolate V245; sub-cultures 7 (lane 5), 5 (lane 6), 3 (lane 7) and 1 (lane 8) of isolate V208 and sub-cultures 11 (lane 9), 9 (lane 10), 7 (lane 11), 5 (lane 12) and 3 (lane 13). (b) formic acid extracts (as in (a)) subjected to perfomic acid treatment. M (protein markers) **187**

CHAPTER 1

General Introduction

1.1 BIOLOGICAL CONTROL OF INSECT-PESTS

DeBach (1964) has defined biological control as the action of parasites, predators and pathogens in maintaining another organism's density at a lower average than would occur in their absence. This definition remains most appropriate since it is simple and yet traditional. A great number of natural enemies of invertebrates, weeds, plant pathogens and even some vertebrates have been identified and new agents are continuously being found (Van Driesche & Bellows, 1996). However, throughout this chapter, literature published on fungal pathogens as biocontrol agents (BCAs) for the control of insect-pests will be reviewed. Control of insect-pests by fungal BCAs has been a topic of several review articles, to which the reader is referred (Latgè & Moletta, 1988; McCoy *et al.*, 1988; McCoy, 1990; Ferron *et al.*, 1991; Roberts & Hajek, 1992; Tanada & Kaya, 1993; Hajek & St Leger, 1994; Boucias & Pendland, 1998; Wraight & Carruthers, 1999; Butt & Copping, 2000; Lacey *et al.*, 2001; Butt *et al.*, 2001a).

Fungi are the most common and widespread microbes causing diseases in virtually all insect orders. They often induce epizootics and thus decimate insect populations (Hajek & St Leger, 1994). Unlike bacteria and viruses, fungi do not have to be ingested to invade their host. Instead, they enter the host by breaching its integument. This characteristic makes fungi the most important pathogens among sucking arthropods such as aphids, which feed by sucking plant sap and do not ingest pathogens. Also, fungi are important for the control of coleopteran species, since viral and bacterial

diseases are very rare among beetles (Hajek & St Leger, 1994; Samish & Rehacek, 1999). Nevertheless, fungi could be successfully used against non-feeding stages of pests (e.g. eggs, pupae) or subterranean pests (e.g. root feeding insects). Most entomopathogenic fungi belong to the family Entomophthoraceae within the sub-division Zygomycotina or in the Deuteromycotina. Although over 700 spp. of entomopathogenic fungi are known to date, only a few have been, or are being, developed for the control of insects (Roberts & Hajek, 1992) (Table 1.1).

The most commonly investigated entomopathogenic fungi are *Metarhizium* spp. and *Beauveria* spp. These fungi are particularly effective against terrestrial insects due to their wide geographic spread and host range as well as their ability to germinate in relatively low humidities (Hall & Papierok, 1982; Hajek & St Leger, 1994). Furthermore, they can be easily cultured *in vitro*, either in liquid or on semi-solid and solid substrata (Butt & Goettel, 2000), resulting in large quantities of blastospores/conidia. The production of conidia that possess thick strong cell walls is also a favourable characteristic, which greatly contributes to production efficiency and storage stability and is one of the main reasons for choosing conidia as a propagule for most commercial formulations.

Table 1.1. Host range of the most common entomogenous fungi (a general list).

Fungal species	Host
Division Zygomycotina	
Conidiobolus obscurus	Homoptera (aphids)
Entomophaga aulicae	Lepidoptera
Entomophaga gryli	Orthoptera
Entomophthora muscae	Diptera (flies)
Entomophthora thripidum	Thrips
Erynia neoaphidis	Homoptera (aphids)
Massospora cicadina	Orthoptera (cicada)
Neozygites fresenii	Homoptera (aphids)
Zoophthora radicans	Hemiptera and Lepidoptera
Division Deuteromycotina	
Aschersonia aleyrodis	Whitefly, scales
Beauveria bassiana	Wide host range
Beauveria brongniartii	Cockchafers and sugarcane borer
Hirsutella thompsonii	Spider mites, citrus mites
Metarhizium album	Homoptera
Metarhizium anisopliae	Wide host range
Metarhizium flavoviride	Orthoptera
Nomuraea rileyi	Lepidoptera
Paecilomyces farinosus	Coleoptera, Lepidoptera
Paecilomyces fumosoroseus	Wide host range
Tolypocladium cylindrosporum	Mosquitoes
Verticillium lecanii	Wide host range

1.2 *METARHIZIUM ANISOPLIAE* AS A BIOCONTROL AGENT

Metarhizium anisopliae (Metschin.) Sorokin (division Deuteromycotina, class Hyphomycetes) has been isolated from about 200 host species among Coleoptera, Lepidoptera, Orthoptera and Hemiptera. It is a widespread, soil-borne pathogen of medical and economic importance (Roberts, 1973; McCoy *et al.*, 1988). Tulloch (1976) recognized two varieties of *M. anisopliae*, var. *anisopliae* Tulloch and var. *major* (Johnston) Tulloch. Both varieties are easily recognized by the long chains of ellipsoidal or cylindrical, with a slight central constriction, conidia issuing from phialides. Hydrophobic conidia are usually coloured dark green, however, a few conidial colour variants (from light green to dark brown) of *Metarhizium* spp. have been reported (Rombach *et al.*, 1987). Conidial size of *M. anisopliae* ranges from 3.5 to 9.0 μm long for *M. anisopliae* var. *anisopliae* and from 9.0 to18.0 μm long for *M. anisopliae* var. *major*. The large spored *M. anisopliae* var. *major* is more homogeneous than var. *anisopliae*, which shows high isozyme polymorphism (Riba *et al.*, 1986).

As early as 1879, *M. anisopliae* was successfully used in Russia by Metschnikoff (Van Driesche & Bellows, 1996) to control pests such as *Anisoplia ustriaca* (wheat cockchafer) and *Cleonus puntiventris*, the sugar beet curculio. In the last decade, some isolates of this organism have been developed into commercially available products (Table 1.2).

Table 1.2. Fungi registered and commercially marketed as biological control agents of pests (revised from Butt & Copping, 2000; Wraight *et al.*, 2001).

Product	Target	Producer
BIO 1020	Black vine weevil	Germany
BioGreen	Scarab Larvae on pastures	Australia
Metabiol (Metaquino)	Spittlebug	Brazil
Metarril	Sugar-cane spittlebug	Brazil
Cobican	Sugar-cane spittlebug	Venezuela
Meta-Spin	Pepper weevil, boll weevil, Sugar-cane borer	Mexico
Metarhizium Schweizer	White grubs	Switzerland
Bio-Path	Cockroaches	USA
Bio-Blast	Termites	USA

1.3 POTATO-PEACH APHID (*MYZUS PERSICAE* (SULZER))

There are more than 500 species of aphids in the British Isles (Blackman, 1974). However, this section focuses on one of the most common polyphagous aphids, *Myzus persicae*, which is considered to be one of the most economically important agricultural and horticultural insect pests in the temperate climatic zones.

Myzus persicae (Aphidinae: Marcosiphini) Sulzer (Plate 1.1) is commonly known as the peach-potato aphid or green peach aphid. Adult apterae have a small to middle-sized body of pale-green, pale-yellow-green, grey-green, mid-green, pink, and red or almost black colour. Alatae have a black central dorsal patch on the abdomen (Blackman & Eastop, 2000). The summer forms are all females. They are *viviparous* (give birth to live young) and produce their nymphs from unfertilized ova by *parthenogenesis*, a method of reproduction that enables them to multiply at tremendous rate. By producing live young,

the aphids avoid a time-consuming egg-stage, and the embryos develop to an advanced condition within the mother's body so that nymphs can commence feeding as soon as they are born. Even before birth, young females carry embryos of the next generation. After birth, the nymphs take two weeks to develop into adults.

Peach-potato aphids feed almost continuously throughout nymphal and adult life on nutrients of healthy plant's sap. The primary host is usually *Prunus persica* or *P. persica* var. *nectarina*, although it may sometimes feed on *P. nigra*, *P. tenella, P. serotina* and peach-almond hybrids. Secondary hosts include over 40 different plant

Plate 1.1. Larvae and adults of oviparous females *Myzus persicae* (Sulzer) on fresh leaves of Chinese cabbage.

families of which many are economically important (Blackman & Eastop, 2000). It has been proposed that there are two types of stimuli in aphid-host-plant selection (Kennedy & Booth, 1951). Flavour stimuli provide information about host plant on which the aphid is to alight. However, the aphid has to make an initial test probe in order to decide whether the plant is accepted or rejected. Nutrient stimuli, on the other hand, tell the aphid about the physiological condition of the plant or the age of a leaf, information relevant to its value as food. *Myzus persicae* have an amazing ability to discriminate between different diets and most rapidly feed on diets with optimum concentrations of sucrose and amino-acids (Mittler, 1970; Harrewijn & Noordink, 1971) which are essential for protein synthesis, growth and reproduction. Sugary droplets known as 'honeydew' are excreted through an aphid's anus equal in quantity to the plant sap that is imbibed. However, the content of honeydew is different from that in plant sap (Mittler, 1976). For example, sucrose, when ingested, is converted into monosaccharides and trisaccharides by invertase enzymes. Being relatively large molecules, the trisaccharides tend to pass through the aphid's gut unabsorbed. Honeydew also contains most of the amino acids and amides that are found in plant sap, though in smaller quantities. *Myzus persicae* is likely to be found on older, senescing leaves, where chemical compounds are broken down to enrich the sap flow to the growing points.

Individually, aphids do not impose any threat to a healthy growing plant. Acting together in sufficient numbers, they can cause a devastating damage to crops worldwide. They are considered to be one of the most important pests of UK crops, causing damage

directly by feeding (Mallot & Davy, 1978) and indirectly by transmitting plant virus diseases such as virus yellows in beet crops, leaf roll virus and virus Y in potato crops (Kennedy *et al.*, 1962; Eskanderi *et al.*, 1979). Furthermore, the sugar-rich honeydew secreted by aphids encourages premature leaf ageing (Rabbinge *et al.*, 1981) and growth of saprophytic and pathogenic fungi on crop plants (Bergstrom *et al.*, 1982) reducing both yield and quality.

In addition to their direct involvement as virus transmitters, aphids have large reproductive rates and their wide range of host plants means that they are difficult to control. Selective aphicides have been used to reduce populations to below damage threshold levels (Schepers, 1989). If they are to be used as sole control agents for aphids that have several generations during a crop cycle, they will have to be applied repeatedly because of the reproductive capacity of the pest. The extensive use of organophosphorus, organochlorine, carbamate and pyrethroid insecticides has led to the development of resistance in more than 20 aphid species, not least in *M. persicae* (Hurkova, 1973; Sawicki & Rice, 1978; Baker, 1978; Attia & Hamilton, 1978; Bauerfeind & Chapman, 1985; Devonshire, 1989; Foster *et al.*, 1998). This alone represents one of the major threats to the future success of chemical control of aphids. Nevertheless, inherent toxicity of many insecticides, coupled with their potential to be environmentally hazardous, are the main reasons why they have been largely abandoned.

There are many other, non-chemical, methods that can contribute to aphid control. For example, breeding or induction of resistance or tolerance in crop plants, and the

adjustment of cultural practices so as to favour a low incidence of pests or a high incidence of natural enemies (Carver, 1989) could be adopted. However, these methods become less attractive to growers where economics demand specialisation and intensification. In this case, interests shift towards other means of control such as habitat manipulation such as placement of artificial food as supplements or attractants for natural enemies, genetic manipulation of pest populations such as the inundative release of sterile males, or the use of pheromones in order to modify pest behaviour and insect growth regulators. Although the use of classical biological control agents such as the use of natural enemies for the control of aphids is the most successful to-date (Carver, 1989), exploration of other potential biocontrol agents is needed.

In a recent review, Inglis *et al.* (2001) presented examples where efficacious management of aphid pests in greenhouses was achieved by exploiting entomopathogenic Hyphomecetes as BCAs. The controlled environment in greenhouses could be manipulated in such a way as to favour the initiation and development of fungal diseases of aphids. For example, a single application of *Verticilium lecanii* conidia, just before covering plants with polyethylene, was observed to provide satisfactory control of *M. persicae* (Hall & Burges, 1979), one of the most economically important pests of chrysanthemums throughout the world. Significant research into the development of *V. lecanii* has led to the commercial production of the mycoaphidicide, Vertilec® (Hall, 1981). Although several isolates of the entomogenous fungus *M. anisopliae* have been shown to be pathogenic to the aphid species *M. persicae* and

Lipaphis erysimi (Butt *et al.,* 1995) under laboratory conditions, much more research and development is needed to further their exploitation in the field.

1.4 THE CONCEPT OF PATHOGENICITY AND VIRULENCE

Davis (1990) defined 'pathogenicity' as a capacity of micro-organisms to cause disease. The term 'virulence' is generally used to note variations in degree of pathogenicity. In other words, virulence is a measure of the severity of disease as assessed by reductions in host fitness following infection (Read, 1994). These abilities are determined by a variety of factors such as the physiology of the insect, physiology of the fungus and the environment (Inglis *et al.*, 2001). In addition, evolution of virulence gives us every reason to think that natural selection has the potential to affect disease severity (Read, 1994). If host and pathogen fitness is affected by virulence, then it is likely that genes determining disease are also under strong selection. Furthermore, there is clearly genetic variation on which selection can act, since virulence polymorphisms are common (Sibley & Boothroyd, 1992). Virulence determinants are often encoded by phage, plasmids and transposons (Levin & Eden, 1990) and artificial selection can maintain or reduce virulence (Bull *et al.,* 1991). The effects of migration, mutation or recombination could also explain genetic variation in virulence. The diversity of the micro-organisms that cause disease, coupled with individual host differences, make an understanding of pathogenesis difficult. It is further complicated by variability among strains within a given species (Cutler, 1991). Key factors to the successful development of fungi as BCAs are the identification of virulent isolates and an understanding of the underlying

mechanisms of virulence and attenuation (the reduction in virulence). Highly virulent isolates encompass two key attributes: the ability to kill in a relatively short period of time (i.e. low LT_{50}: the time taken to kill 50% of the population) and the ability to cause high mortality at relatively low doses (i.e. low LC_{50}: concentration required to kill 50% of the population). Failure to maintain a consistent level of virulence or the inability to predict fungal infectivity has hindered progress. It is, therefore, of paramount importance to understand the phenomena of virulence and attenuation.

1.4.1 Attenuation of virulence

Virulence may decrease when pathogenic micro-organisms are kept in culture for extended periods of time or when the pathogen is passed through culture media more then once. This partial or complete loss of virulence is known as attenuation (Fox & Jacques, 1958; Kawakami, 1960; Schaerffernberg, 1964; Nagaich, 1973). Although virulence could be fully restored by passaging the pathogen through an insect-host (Fargues & Roberts, 1983; Morrow *et al.*, 1989; Prenerova, 1994), it is also possible that this phenomenon might be irreversible (Hall, 1980). In this case, attenuation requires explanations, not only in terms of cellular and molecular mechanisms such as communication at the cellular level, effect of environmental conditions on expression of cell surface components (Cutler, 1991; Kobayshi & Cutler, 1998; Cutler, 2001) or genes responsible for virulence, but also in terms of virulence factors such as enzymes with cuticle degrading properties (Table 1.3); toxins and their role in pathogenesis

Table 1.3. Cuticle-degrading enzymes of entomogenous fungi.

Enzyme	Fungi	Specificity	Regulation	pH	pI	MW (kDa)	Reference
Subtilisin subclass (Pr1) serine endoprotease	*M. anisopliae*	Chymoelastase	C,N-repression; specific protein induction	8	4 isoms 10.2; 9.8; 9.3; 9.0	4 isoms 30.2; 28.5; 29.5; 31.5	St.Leger *et al.*, 1987a, 1987b, 1994a; Paterson *et al.*, 1994b; Joshi *et al.*, 1997; Gillespie *et al.*, 1998; Segers *et al.*, 1999
Serine endoprotease (Pr2)	*M. anisopliae*	Trypsin-like	C,N-repression; non-specific protein induction	8	3 isoms 4.4; 4.9; 5.2	28.8	St.Leger *et al.*, 1987a, 1987b, 1994a, 1996a; Paterson *et al.*, 1993; Cole *et al.*, 1993; Smithson *et al.*, 1995; Gillespie *et al.*, 1998; Segers *et al.*, 1999
Cysteine endoprotease (Pr4)	*M. anisopliae*	Trypsin-like	?	?	4.6	26.7	Cole *et al.*, 1993
Metallo aminopeptidase	*M. anisopliae; A. fumigatus*	Post alanyl	N-repression; weak C-repression; protein induction?	7	7.3	42	St.Leger *et al.*, 1993, 1994a, 1995
Carboxypeptidase	*M. anisopliae*	?	?	6.8	9.97	30	St.Leger *et al.*, 1994b; Joshi *et al.*, 1999
Esterase	*M. anisopliae; B. bassiana*	?	?	8	?	?	St.Leger *et al.*, 1986; Kosir *et al.*, 1991
N-Acetylglucosaminidase	*M. anisopliae*	NAG_{2-4}	Constitutive	7	4.51	45	St.Leger *et al.*, 1993
Serine dipeptidylpeptidase	*M. anisopliae*	Post prolyl	N-repression; weak C-repression; protein induction?	8	4.01	74	St.Leger *et al.*, 1993
Lipase	*B. bassiana*	?	?	6	?	?	Kosir *et al.*, 1991
Chitinase	*M. anisopliae; B. bassiana; A. flavus*	> NAG_3	C-repression; NAG induction	5 5.3	5.4-8.6	33 60	St.Leger *et al.*, 1986, 1991, 1996b; El-Syed *et al.*, 1989; Bogo *et al.*, 1998; Kang *et al.*, 1999
Amylases	*M. anisopliae*	?	?	?	?	?	Da Silva *et al.*, 1989; Valadares & Azevedo, 1996

or maybe transposons. The latter are being identified in an increasing number of filamentous fungi (Daboussi *et al.*, 1992; Kistler & Miao, 1992; Kück *et al.*, 1998).

It is well known that when vegetative growth ceases, hyphae senesce and autolyse. This usually occurs when exogenous nutrients are exhausted. Sometimes fungi exhibit senescence long before nutrients become limiting or secondary metabolites reach a harmful level. This is probably due to the expression of cytoplasmically inherited lethal genes. For example, Esser *et al.* (1984) found a ring plasmid, which was incorporated into the mitochondrial genome of the ascomycete *Podospora anserina.* Self-replication of this plasmid coincided with the cell ageing, resulting in rapid senescence and death. Wright & Cummings (1983) and Bainbridge (1987) suggested that such plasmids might be transposable elements capable of movement from one location to another within the fungal genome, resulting in a sudden and drastic physiological event. In parasites, this may manifest changes in pathogenicity. Maurer *et al.* (1994) have reported the discovery of a repetitive DNA element presenting the characteristics of a transposon in *Beauveria bassiana.* This mobile genetic element was cloned as an insertion, inactivating the gene encoding nitrate reductase.

Clarkson (1996) has suggested starvation as being a key environmental signal for the switch from saprophytic to a pathogenic mode of growth, possibly following depletion of nutrients on the insect cuticle. Likewise, St Leger (1992a) found that when rapidly growing mycelia of *M. anisoplia* (ME1) were transferred to a nutrient poor media such as chitin-containing media, it responded within 3 h by producing Pr1, a major cuticle-

degrading enzyme. This indicates that nutrient levels co-ordinate regulation and expression of gene products for both morphological development and enzymatic degradation of cuticular proteins. The expression of the cell surface components in many fungi of medical importance such as *Candida albicans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* were also affected by environmental and nutritional conditions (Hazen & Hazen, 1987; Sweet & Douglas, 1991; Klimpel & Goldman, 1987, 1988; Klein *et al.*, 1994), growth phase and morphological form (Kambe *et al.*, 1991). Molinari *et al.* (1993) hypothesized that the pathogenic functions of cell surface variability help the fungus to escape host immune recognition and alter its adherence properties. For example, "switching phenomenon" (a change of its surface from hydrophobic to hydrophilic and back) in *C. albicans* is shown to relate to glycosylation/deglycosylation of the phosphomannoproteins and correlates with pathogenesis (Slutsky *et al.*, 1985, 1987).

Many hyphomycetes and entomophthoralean fungi reproduce asexually. Although teleomorph stages are known for some, it is the anamorph which predominates. The possibility of recombination or heterokaryosis between strains could also explain genetic variability within and between fungal species. For example, Messias & Azevedo (1980) and Al-Aidroos (1980) have demonstrated genetic recombination through the parasexual cycle under laboratory conditions (*in vitro)* for *M. anisopliae*. Moreover, when larvae of *Phaedon cochleariae* were inoculated with two strains of *M. anisopliae*, one out of 53 insects seemed to be co-infected, suggesting diploidization or recombenation at high frequency (10-33%) (Leal-Bertioli *et al.,* 2000). Viaud *et al.*

(1998) also reported genetic exchange between two auxotrophic strains of *B. bassiana* and *Beauveria suphurescens*.

There are a number of approaches to identify virulence factors in entomopathogens, which could be compared quantitatively or qualitatively. These approaches are:

1. Bioassays with target insect (e.g. LT_{50} and LC_{50} values);
2. Biochemical (the use of specific substrate for enzyme activity by measuring absorbency);
3. Classical genetic approach (mutagenesis, genetic recombination);
4. Molecular biological techniques including Polymerase Chain Reaction (PCR)-amplification of any piece of DNA, Random Fragment Length Polymorphisms (RFLP) - used to investigate phylogeny, Random Amplification of Polymorphic DNA (RAPD) - methods for evaluation of the genetic information, allozyme techniques - used to study isozyme variations within fungal spp., Reverse Transcriptase (RT)-PCR used to detect and quantify gene expression or to investigate the relationship between gene expression and virulence, transposon-tagging method used to analyse gene function and genes disruption;
5. ELISA- may provide a direct comparison of virulent and attenuated strains.

1.5 NUTRITION AND PATHOGENESIS

Minimum requirements for sources of carbon, nitrogen, phosphorus and other minerals, vitamins and other growth factors vary widely for different fungi. It is reasonable to assume that laboratory-defined substrate requirements have direct relevance to econutritional behaviour. It is very important to make a distinction between reserve and structural compounds in terms of their availability to fungi in both time and space. Starch, inulin, glycogen and lipids are abundant in plants and animal cells and can be readily utilised. By contrast, plant and microbial cell walls and extracellular structural components of plants and animals have greater persistence, and so become available over a longer period of time.

Recent work by St. Leger *et al.* (1997) demonstrated the ability of a range of fungal species including saprophytes (*Neurospora crassa, Aspergillus nidulans*), phytopathogens (*Verticillium albo-atrum, Verticillium dahlia, Nectria haematococca*), a mushroom pathogen (*Verticillium fungicola*), an opportunistic insect pathogen (*Aspergillus flavus),* an opportunistic human pathogen (*Aspergillus fumigatus*) and entomopathogenic fungi (*M. anisopliae, B. bassiana, V. lecanii*) to readily adapt to the requirements of their ecological niches. All of the pathogens showed enzymatic adaptation to the polymers present in the integuments of their particular hosts. For example, plant pathogens produced high levels of polysaccharide, cellulose, xylan and cutin degrading enzymes but no elastin or mucin degrading enzymes and very little or no chitinases. In contrast, entomopathogens such as *M. anisopliae, B. bassiana,*

V. lecanii and the mushroom pathogen *V. fungicola*, secreted very low levels of polysaccharidases and high levels of broad spectrum protein-degrading enzymes (including elastin and mucin). The saprophytes and the opportunistic pathogens were able to produce the broadest spectrum of protein and polysaccharide degrading enzymes, indicating their less specialised nutritional status.

Many fungi possess mechanisms that provide them with the versatility to exploit many environments (St Leger & Screen, 2000). For example, *A. fumigatus* is a saprotroph but may sustain itself on a human host by producing depolymerases and colonise the respiratory tract. Such mechanisms of pathogenicity may have evolved to adapt this fungus to life as a saprobe.

There is no ideal balance of the different nutrients in the environment, but if, for example, carbon compounds are present in excess, they will be taken into the cell and converted into a form suitable for storage or secreted as surface carbohydrates. Such kind of reserves may be mobilised and used as a source of energy or for biosynthesis (Cooke & Whipps, 1993).

1.5.1 Endogenous reserves in fungi

Spores contain a variety of nutrient reserves which could be located in membrane-bound storage vacuoles, the cytoplasm in solution or as droplets or granules, or even in the cell wall (Cooke & Whipps, 1993). The major reserves of fungi are carbohydrates including

polyols (acyclic sugar alcohols), trehalose, glycogen, lipids and occasionally starch (Blumental, 1976; Jennings, 1994). These can be derived from glucose or fructose either via glycolysis or the pentose phosphate pathway (Jennings, 1995). Interestingly, these reserves are synthesized by one pathway but degraded by another, a system that gives good control of the whole process of biosynthesis. These pathways are very much dependent on the growth stage, environmental conditions and nutrition of the fungus (Cooke & Whipps, 1993).

The quantity of individual endogenous reserves is determined by the nature of the external medium such as carbon or nitrogen (Rfyffer & Rast, 1980; Lane *et al.*, 1991b; Hallsworth & Magan, 1994a) and osmotic potential of the medium (Hallsworth & Magan, 1994b; Ramos *et al.*, 1999). Glycerol, arabitol, erythritol and mannitol, as well as being osmoregulators (organic solutes accumulated under water stress), are thought to serve the following roles: (a) as energy (carbohydrate) reserves which enter into cell metabolism as soon as they are required, (b) as translocating compounds, (c) in co-enzyme regulation and (d) helping to maintain turgor (Jennings, 1995). High concentration of polyols enables fungal growth at low water availability by reducing intracellular water activity (a_w) and by acting as 'compatible solutes' which do not destabilize or inhibit enzyme activities at metabolic level (Brown, 1976, 1978; Yancey *et al.*, 1982, Carpenter & Crowe, 1988; Jennings & Burke, 1990; Rudolph *et al.*, 1993). If a fungus is lacking polyols, the amino acid proline is synthesized in response to lowered water potential. This organic compound has very little effect on enzyme activity (Luard, 1982). Polyols not only aid survival but also have a function during re-wetting,

which enables the thallus to survive the loss of organic solutes and mineral ions during resaturation-respiration period. It is also suggested that polyols may play a role in controlling proton availability (Jennings, 1984).

The disaccharide trehalose can replace water in dehydrated phospholipid membranes at low a_w (Crowe *et al.*, 1984; Tsvetkov *et al.*, 1989; Crowe & Crowe, 1993) by inhibiting transition of the lipid crystalline phase to the gel phase and so stabilises enzyme structure during desiccation (Carpenter & Crowe, 1988; Colaco *et al.*, 1992). Reserves of trehalose can be converted directly to glucose by trehalase. It has also been reported that increased trehalose content in microbial propagules enhances desiccation tolerance and germination at low water availability (Panek, 1963; van Laere *et al.*, 1987). This disaccharide is also synthesized in response to nutrient deprivation and exposure to toxic chemicals acting as a general stress protectant in the cytosol (Wiemken, 1990). In addition to the mentioned reserves, fungal cells may contain lipid reserves (carbon source) which are partially utilized during dormancy, whereas metabolism of other reserves may be prevented until germination commences.

1.5.2 Ecophysiological manipulation of endogenous reserves in fungal BCAs

Extreme environmental factors restrict or prevent fungal development. However, in many situations, fungi which possess appropriate physiological characteristics or which can adapt through a temporary alteration in their development, could tolerate these extremes. Such natural abilities could be easily exploited when looking for ways of

improving strains or at least trying to predict their behaviour. At present, very little attention has been given to the manipulation of spore physiology as a means of improving inoculum quality and its stability during production and storage life.

Studies of some entomogenous fungi that use carbon and nitrogen sources have demonstrated that concentrations of C and N have a significant effect on the carbohydrate content of mycelia and blastospores of *B. bassiana* and *P. farinosus* produced in liquid culture, and on the sugars and sugar alcohols of *M. anisopliae, B. bassiana* and *P. farinosus* conidia grown on solid medium (Bidochka *et al.,* 1990; Lane *et al.,* 1991b; Hallsworth & Magan, 1994a). Furthermore, the optimum quantities of such accumulated sugars and low-molecular-weight polyols (Hallsworth & Magan, 1994a) varies with time (7 to 21 days) at constant temperature of 25° C.

A significant increase of trehalose concentration (Harman *et al.,* 1991) has been observed in fungal mycelium and spores of *Trichoderma harzianum,* when grown on media amended with polyethylene glycol (PEG) 8000. Polyol and trehalose content of conidia of three entomopathogens (*B. bassiana, M. anisopliae* and *P. farinosus*), grown on media modified with potassium chloride (KCl) to give a range of water potentials, have also been shown to undergo considerable changes (Hallsworth & Magan,1994b). These, and other recent studies (Pascual *et al.,* 1996; Jackson *et al.,* 1997; Frey & Magan, 1998; Teixido *et al.*, 1998a, 1998b) have shown that endogenous content of sugars and sugar alcohols in a number of fungal BCAs could be successfully modified using the approach described above.

Improved viability, germination and growth capacities of modified inocula over a wider range of water-stress conditions have been demonstrated in a few studies. For example, conidia of *M. anisopliae, B. bassiana* and *P. farinosus* modified with KCl and glycerol (Magan, 2001) and yeast cells of *Candida sake* modified with either glucose or glycerol (Teixido *et al.*, 1998 b) showed greater viability over low ranges (0.95-0.93 a_w) of water availability than unmodified propagules or yeast cells under the same conditions. Schisler *et al.* (1991) also demonstrated an improved germination and efficacy of conidia of the mycoherbicide *Colletotrichum truncatum* against *Sesbania exaltata* by producing inocula in medium with low carbon/nitrogen ratio. Although manipulation of endogenous reserves in conidia of *Epicoccum nigrum* and *Ulocladium atrum* did not result in enhanced germination, a significant improvement of germ-tube extension was achieved after 24 hr of incubation under water-stress environment (Pascual *et al.*, 1998).

Improved ecological fitness studies have also been undertaken in order to identify factors which influence the efficacy of prospective BCAs under field conditions (Jin *et al.*, 1991; Hallsworth & Magan, 1994c; Jackson *et al.*, 1997; Teixido *et al.*, 1998a, b, c; Frey & Magan, 1998; Pascual *et al.*, 1998; Anderson, 2000). Results from such studies have clearly shown that culturing inocula on a specific artificial media significantly influences establishment in the natural environment and effectiveness against targeted insects, weeds and diseases. Thus, the way in which inoculum is produced should be considered when developing an ecologically competent BCA for field use.

An ecophysiological manipulation strategy which translates into the development of stable, conserved or improved quality inocula under a range of environmental conditions could be applied to the majority of fungal species. Unfortunately, how endogenous components of modified organisms relate to their pathogenicity or virulence still remains unknown. Further studies are, therefore, required to evaluate the roles of endogenous reserves in underlying mechanisms of pathogenicity.

1.5.3 Occupation of the exoskeleton

During the Devonian period, when the first terrestrial insects appeared on earth, it would have been possible that insect chitin, as one of the components that increasingly enriched organic debris, encouraged the emergence of ancestrial chitinoclastic fungi. From that point, fungal species capable of utilising the exoskeleton of living insects have evolved (Evans, 1988).

The cuticle of an insect is complex and varies according to species. Furthermore, it also undergoes changes during the development of individuals. The epicuticle is hard, hydrophobic and lacks chitin, consisting mostly of waxes, tanned proteins, polyphenols and highly polymerised lipids as well as fatty acids, which may have antifungal properties (Smith & Grula, 1982; Butt *et al.*, 1995; Ibrahim *et al.*, 1999). The procuticle is thicker and is rich in chitin fibrils embedded in a protein matrix, lipids and quinones (Hillerton, 1984). The mechanical properties of insect cuticle very much depend on the proportion of these two main components, the nature and level of hydration of these

proteins and sclerotisation. Since the degree of sclerotisation is determined by cross-linking of the proteins by quinones, the latter have a particularly strong influence on penetrability. Since water extrusion accompanies sclerotisation, water availability in addition to phenolic cross-links may be a factor limiting penetration (Charnley, 1992). Epicuticular lipids may also reduce penetration of chemicals and toxics as well as infectious microorganisms (Juàrez, 1994; Lecuona *et al.,* 1997).

Fungus-insect iteractions although complex could be presented in a simplified diagram (Figure 1.1), which is discussed over the next few sections.

1.5.4 Attachment

For many fungal pathogens, pathogenesis begins when the fungus makes initial contact with the insect and adheres to its cuticle. Although the forces responsible for the interaction between fungal elements and insect cuticles are not well understood, it is generally proposed that the adherence process involves two steps. The initial step involves passive non-specific (hydrophobic/electrostatic and hydrophilic forces) or specific interactions (surface antibodies, glycoproteins, glucans, sugar, etc.) (Fargues, 1984; Boucias & Pendland, 1991; Rath *et al.,* 1995) followed by more permanent attachment through a combination of enzymes and mucilage (Butt, 1990; Bidochka *et al.*, 1997). The second step is an active process, which continues during germination and appressoria differentiation (St Leger *et al.*, 1989).

Infective propagule (spore)

↓

Cuticle

Attachment→ (hydrophobic/hydrophilic forces→lectins→enzymes→mucilage)

Signalling reactions trigger germination→growth of fungal germ tube (use of energy reserves)→switch to pathogenic mode of growth

↓

→ Release of fungistatic compounds from the cuticle

Cuticle substrates, morphology, and topography cue appressorium formation

↓

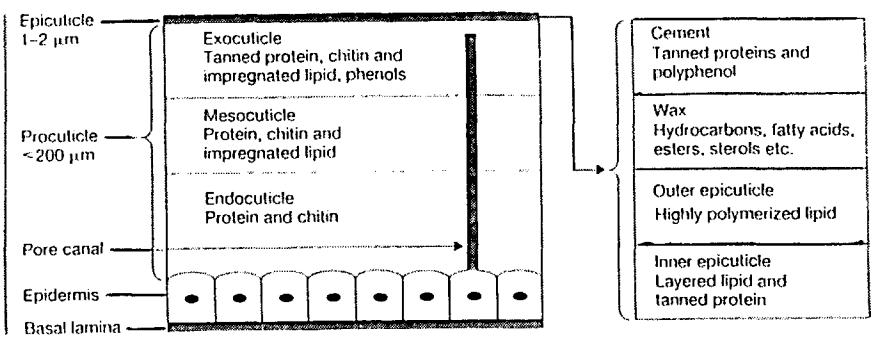
Release of specific to cuticle substrate degrading enzymes + mechanical forces

↓

ACTIVATION (signalling-receptor interaction)→induction and release of multiple enzymes, ATP/GTP binding

↓

Cuticle penetration



Host immune response (melanisation, phagocytosis, nodulation or encapsulation)

↓

Secondary metabolites and toxins production

↓

Colonisation and replication of fungus

↓

Hydrolysis of hosts' proteins, cell lyses

↓

Death of host

Figure 1.1 Schematic representation of pathogen-host interaction in relation to the link between nutrition and pathogenesis.

Dry, hydrophobic conidia of *M. anisopliae, B. bassiana, Nomuraea rileyi* and *P. fumosoroseus* are covered by layers of interwoven bundles of extremely hydrophobic rodlets which are composed of proteins (hydrophobins), lipoproteins, glycoproteins or polysaccharides (Boucias & Latgé, 1986; Boucias *et al.,* 1988; Boucias & Pendland, 1991; Bidochka *et al.,* 1995; Jeffs *et al.,* 1999). This rodlet layer attaches conidia to host cuticle by strong binding forces involving hydrophobic and, to a lesser extent, electrostatic interactions. Spores with rugose surfaces are more hydrophobic than those with smooth surfaces (Jeffs *et al.,* 1999). Surface hydrophobicity of a host also influences adhesion of fungal pathogens. For example, more conidia of *M. anisopliae, B. bassiana* and *N. rileyi* was shown to bind to the neonate larval cuticle than to the less hydrophobic 4th instar larval cuticle (Boucias & Pendland, 1991). Adhesion of conidia of *B. bassiana, M. anisopliae* and *N. rileyi* was also shown to be affected by cuticular topography and chemical properties of the epicuticle (Boucias *et al.*, 1988). Cuticular areas with short spines were more preferable for attachment than any other body region. *Metarhizium anisopliae* conidia were more firmly attached to the epicuticle in the intersegmental folds than to the smooth exposed sclerite epicuticle (Boucias & Pendland, 1991). In contrast, the wet hydrophilic conidia of *Verticillium, Aschersonia and Hirsutella* spp., lack a well organized outer rodlets layer, instead possessing a mucilaginous coat that aids in adherence to substrates (Hajek, 1997). Mucilage also facilitates rain splash dispersal for some fungal species (Gregory *et al.*, 1959), modifies epicuticular waxes (Wraight *et al.,* 1990) and protects conidia from sunlight and desiccation (Dickinson, 1986).

Carbohydrate-binding glycoproteins, which have been identified on the wall surfaces of *M. anisopliae, B. bassiana* and *N. rileyi* conidia and in the mucus of the ballistospores of *Conidiobolus obscurus* (Boucias & Latgé, 1986) could potentially provide specific linkages between insect host and a pathogen. Such a biphasic system is well established in *Candida*-human cell (Cutler, 2001; Canderone *et al.,* 2000), fungus-nematode and fungus-fungus interactions (Nordbring-Hertz & Chet, 1986). However, competitive inhibition studies (Boucias *et al.,* 1988) have failed to demonstrate the importance of wall-surface-associated lectins in the attachment process of entomopathogenic hyphomycetes.

Adhesion to host cuticle is a pre-requisite for successful invasion. For example, poor attachment of *M. anisopliae* conidia to the larval siphon of *Culex pipiens* was attributed to hypovirulence (Al-Aidroos & Roberts, 1978; Hajek & St. Leger, 1994). Spore attachment may also relate to host specificity since spores of *Coelomyces sporophorae* have been shown only to attach to susceptible mosquitoes (Zebold *et al.*, 1979), *M. anisopliae* conidia only adhered to the cuticles of some scarabs (Fargues, 1984) and *Erynia radicans* conidia only adhered to certain leafhopper species (McGuire, 1985). Conversely, reflecting the importance of non-specific hydrophobic forces in the majority of adhesion, conidia equivalently bind to host and non-host surfaces (Boucias & Latgé, 1986; Boucias & Pendland, 1991).

1.5.5 Germination

Given its physicochemical variability and its antifungal properties, the nutritional potential of the cuticle would suggest that different fungal species require different germination stimuli. For example, *B. bassiana* and *M. anisopliae* will germinate on a range of insect cuticles, whereas species with a more restricted host range such as *Nomuraea rileyi* and *Erynia variabilis* are more fastidious (Boucias & Pendland, 1984; Kerwin & Washino, 1986).

Water, temperature, nutrients, light, ions, fatty acids, the biota on the cuticle surface, and the physiological state of the host are factors that influence spore germination and behaviour (Cooke & Whipps, 1993). *Metarhizium anisopliae* and *B. bassiana*, for example, will readily germinate, grow and sporulate at optimal temperatures of between 25 and 32°C and relative humidities ranging from 98 to 100% (Walstad *et al.*, 1970; James *et al.*, 1998). *Entomophthora muscae* (Kramer, 1980) and *Entomophthora planchoniana* (Holdom, 1984), however, are only capable of germination at low humidities. Most entomopathogenic fungi are thought to produce spores and infect at night (Hajek, 1997). For example, it took only 3.4 hours for 50% of *Entomophaga maimaiga* conidia to germinate in the dark but 9 hours was required for the same number to germinate in the light (Hajek *et al.*, 1990). Although, *M. anisopliae* isolate ME1 has a nutrient-independent pre-swelling growth phase of germination (Dillon & Charnley, 1986), this strain along with the other entomopathogens, requires an exogenous nutrient (carbon and nitrogen) source for conidial swelling and germ-tube

formation (Smith & Grula, 1981; Dillon & Charnley, 1985; Kerwin & Washino, 1986). Amino acids and amino-sugars will accelerate germination of hydrated conidia of *M. anisopliae* while polymeric chitin is a poor substrate for germination and growth (Hassan *et al.*, 1989).

While water-soluble nutrients (mainly amino acids) on the surface of *Heliothis zeae* larvae are sufficient for *B. bassiana* germination (Woods & Grula, 1984), the same fungus would not germinate on more sclerotised cuticle of *Dendroctonus ponderosae* without a nutrient supplement (Hunt *et al.*, 1984) suggesting that nutrient availability on the surface of insect cuticle may be limiting. Furthermore, for successful invasion, subsequent prepenetration behaviour must be adapted to the nature of the cuticle surface. For example, many of the strains of *M. anisopliae* which germinated well and produced appressoria in glucose media, were frequently related to the nutrient conditions pertaining to their hemipteran or Lepidopteran hosts (insects producing secretions rich in sugars). Conversely, most lines from coleopteran hosts were dGlc-resistant and germinated poorly on glucose (St Leger *et al.*, 1994a).

Signal molecules, which may or may not be cleaved from the cuticle surface, can have a profound effect on germination and infectivity. St. Leger *et al.* (1989) have reported the existence of six families of GTP- binding proteins in plasma membranes of *Metarhizium,* including separate substrates for pertussis and cholera toxin, both of which were antigenically related to mammalian G proteins. The localisation of GTP-binding proteins on plasma membranes implies an involvement in transmembrane

signalling reactions where the cellular membrane of the organism receives external signals, interprets them, and eventually translates them into reactions. Either or both proteins may be necessary to induce protein synthesis in the parasite. There is strong evidence that Ca^{2+} and cyclic adenosine monophosphate (cAMP) may also be involved in germination of conidia, growth and differentiation (St Leger *et al.*, 1989). However, high levels of appressoria formation was observed in a Ca^{2+} - free medium (+EDTA) indicating that Ca^{2+} uptake is not required for differentiation (St Leger *et al.*, 1990). Simultaneous application of ionophore and exogenous Ca^{2+} increased polar hyphal growth and completely inhibited appressorium formation, suggesting that requirements of Ca^{2+} for differentiation are critical.

St Leger *et al.* (1991) proposed that differentiation in *M. anisopliae*, initiated following a contact-induced change in the potential of germ tube membranes, may be due to disruption of the apical Ca^{2+} gradient necessary for polar growth. In agreement, Magalhaes *et al.* (1991a) reported an external Ca^{2+} requirement for appressoria formation in *Zoophthora radicans.* Intracellular levels of cAMP have been noted to rise in coincidence with the onset of appressorium formation (St Leger *et al.*, 1990).

Excess nutrients may stimulate germination but prevent penetration of the cuticle partly through the expression of the protease Pr1 (St. Leger *et al.*, 1989). Successful germination not only requires assimilation of utilisable nutrients, but also tolerance of any toxic/fungistatic compounds present on the cuticle surface (Smith & Grula, 1982; Butt, *et al.*, 1995; Lecuona *et al.*, 1997; Ibrahim *et al.*, 1999). Presumably virulent

isolates are able to overcome the effect of inhibitors and rapidly respond to the appressoria stimulatory cues.

1.5.6 Cuticle penetration

When germ-tube elongation ceases, it is usually followed by appressorium formation. Both may be initiated by a combination of cuticle substrates, morphology, and topography. Incursion may be affected by a germ-tube (Butt *et al.*, 1995), undifferentiated hypha, and a penetration peg under the appressorium or by penetrant hyphae produced by appressoria. Low concentrations of complex nitrogenous compounds can induce appressorial formation in *M. anisopliae* whilst carbon catabolites can repress it (St Leger *et al.*, 1989, 1992; Magalhhaes *et al.*, 1991b). Appressoria formation can also be triggered by thigmotrophic stimuli (Clarkson, 1996; Hajek, 1997).

Penetration through insect cuticle is achieved by a combination of enzymatic and mechanical forces (Charnley, 1989; Charnley & St. Leger, 1991; St Leger, 1993; 1995). The precise role that individual enzymes play in cuticle penetration and subsequent stages of pathogenesis is poorly understood, although considerable advances have been made in the last few years in our knowledge of cuticle-degrading enzymes (St Leger *et al.*, 1986, 1987a, 1987b, 1988). The structure of insect cuticle is complex and suggests a possible synergistic action of different enzymes in its penetration. Since proteins are the major component of insect cuticle (Locke, 1984), it follows that proteases must play an important role in the penetration process. Evidence for the importance of proteases was

derived largely from studies of their production in infected cuticle associated with cuticle degradation, the effects of protease inhibitors on pathogen behaviour, and by the analysis of protease deficient mutants (Table 1.3). More recently, studies have included cloning, identification, and manipulation of specific protease genes of *M. anisopliae*, particularly those of the subtilisin, Pr1, also produced by many other insect mycopathogens (St Leger *et al.*, 1995). Extracellular enzymes produced by most entomopathogenic fungi solubilise cuticle proteins, which assist penetration and provide nutrients for further growth, and seem to appear in the sequence: esterases, endoproteases (subtilisin-like Pr1a and Pr1b and trypsyn-like Pr2 and Pr4), aminopeptidases and carboxypeptidases with only one chitinase and lipase (Table 1.3).

There is consistent evidence for Pr1 being a pathogenicity determinant. Ultrastructural localisation using immunogold-labelling (Goettel *et al.,* 1989) supports the involvement of Pr1 in cuticle penetration. It has also been suggested that PR1 of *Metarhizium* spp. contributes towards host specificity across different isolates of this fungus (Gillespie *et al.*, 1998). A wide range of entomopathogenic fungi produce Pr1- and Pr2 -like endoproteases including *M. anisopliae, B. bassiana, V. lecanii, Aschersonia aleyrodis* and *N. rileyi.* The regulation of *M. anisopliae* proteases is subject to carbon catabolite and nitrogen metabolite repression (Paterson *et al.*, 1993). However, Paterson *et al.* (1994a, 1994b) reported for the first time that induction of Pr1 was regulated by a specific substrate - insect cuticle.

Western blot analysis with polyclonal antibodies has demonstrated that subtilisin-like proteases differed in serological properties between entomogenous isolates of *Verticillium, Paecilomyces, Metarhizium* and *Beauveria* (Segers *et al.*, 1999). A multiple isoform occurrence in single strains suggests the presence of subtilisin gene families. Thus, diversity between multiple isoforms of subtilisins may prove useful when assessing the virulence of *M. anisopliae* and *B.bassiana.*

Pr2, a fungal serine protease which has a tryptic specificity to basic residues, may be involved in entomopathogenicity (St Leger *et al.*, 1996a). Two Pr2 isoforms (pI 4.4 and pI 4.9), which cleave at the carboxyl sides of positively charged amino acids were identified in *M. anisopliae*. Both isoforms were secreted by appressoria on cockroach cuticle and by the penetrant hyphae within the cuticle.

Joshi *et al.* (1997) have identified genes that were specifically expressed by *M. anisopliae* when conidia made contact with the host insect cuticle using differential display-RT- PCR techniques. Differentially expressed subtilisin - like protease (Pr1B)-encoding gene showed 54% similarity to the well characterised Pr1A subtilisin of *M. anisopliae*. Interestingly, Pr1B possesses double substitution (T220 is replaced by serine and N155 by glycine) in the highly conserved sequences comprising the active sites of subtilisins that were previously thought to be absolutely conserved in all subtilisins. Although it was suggested that these naturally occurring substitutions in Pr1B would reduce catalytic efficiency and would imply strong selective pressure for some unknown structure-function benefits imparted by the changes, this matter needs to

be looked at for further analysis of Pr1B. Recently, Pr1B-encoding gene, similar to *pr1*, was cloned from the mycoparasite *Trichoderma harziamum* (Geremia *et al.*, 1993). This subtilisin-like protease has the same substrate specificity as PR1 and is also induced by specific substrates, in this case by fungal cell walls or chitin.

The genomic *pr1* promoter provides a number of binding sites (CREA - C-catabolite represser and AREA - N-metabolite regulator) for putatively expressed proteins. Such binding sites are proven to be functional *in vitro* where CREA - GST fusion protein of *Aspergillus nidulans* binds to two of the three putative CREA sites in *pr1* promoter of *M. anisopliae*. A fragment DNA from *M. anisopliae* was amplified (crr1) using PCR primers based on the DNA-binding domain of CREA showed 91% identity to CREA, suggesting the presence of a creA homologue in the fungus (Screen *et al.*, 1997). Although uncertainty over crr1 gene as a determinant of carbon levels in Pr1 remains, it is suggested that nrr1 (nitrogen response regulator) gene, a homologue of another putatively expressed protein (areA) might be mediating the nitrogen component of Pr1 (Screen *et al.*, 1998).

Joshi & St Leger (1999) have cloned a novel fungal carboxypeptidase (MeCPA) using RT-DD-PCR. Ultrastructural immunocytochemistry of infected *M. sexta* cuticle revealed that MeCPA participates with concurrently produced endoproteases in procuring nutrients. Amino acid sequence of this enzyme showed 35% identity to human carboxypeptidase A1 and 37% homology with A2, suggesting that MeCPA has an equivalent function to digestive pancreatic enzymes. Solubilising cuticle proteins not

only makes these available as nutrients but also unmasks chitin, which represents a potential barrier to penetration. This explains the late appearance of chitinase *in vitro* (St Leger *et al.*, 1986), and the fact that marked chitinase activity *in vivo* (St Leger *et al.*, 1987a) occurs only when infection is well advanced.

Recently, St Leger *et al.* (1996b) have characterized and localised the chitinases from *M. anisopliae, M. flavoviride* and *B. bassiana* during fungal invasion of *M. sexta* cuticle. Each species produced a variety of N-acetyl-beta-D-glucosaminidases but1,4- beta-chitobiosidases were specifically produced by *M. flavoviride*. Low chitinase production was detected during initial penetration of the host cuticle, however, greater levels of chitinase accumulation were observed in zones of proteolytic degradation. This suggests that release of chitinases is dependent on the accessibility of its substrate. It also suggested that chitinases could be virulence factors in fungal entomopathogenicity and may be used in comparison studies of virulence between *M. anisopliae* (Kramer & Muthukrishnan, 1997). El Sayed *et al.* (1989) showed that virulent and avirulent isolates of *N. rileyi* had similar chitinolitic activity throughout their growth. However, virulent isolates consistently had significantly higher levels of activity at the time of penetration. In contrast, Coudron *et al.* (1984) detected low levels of chitinase activity in germlings of virulent isolates of *B. bassiana, M. anisopliae,* and *N. rileyi.* Kramer & Muthukrishnan (1997) have demonstrated that chitinases as catalysts have a capacity to break down cellular components and as receptors could control cellular adhesion.

Lipids form the third and minor component of the procuticle. It is doubtful that lipases are involved in the penetration process as an attempt to extract true lipases from *M. sexta* infected with *M. anisopliae* failed (St Leger *et al.*, 1987b). In fungi, lipases are generally wall bound and although repressed by the presence of monosaccharides, disaccharides or glycerol, are induced by triglycerides, fatty acids and lecithin. The majority of lipases are glycoproteins, which can be tightly bound to hydrophobic surfaces (e.g. insect cuticle). Paris & Ferron (1984) showed that lipase-negative isolates of *Beauveria brongniartii* were avirulent. Likewise, Jackson *et al.* (1985) reported that in *V. lecanii*, the ability to degrade lipid and protein was not correlated with virulence or avirulence since all isolates produced these enzymes.

Boucias *et al.* (1995) suggested that lectin's main targets are the carbohydrates and amylases which are possible candidates for virulence determinants. Valadares & Avezedo (1996) have also shown that amylases operate in the virulence of *Metarhizium* spp. Da Silva *et al.* (1989) found a better correlation between amylase activity and virulence, and between lipase and virulence for *M. anisopliae*, than between protease and virulence.

St Leger *et al.* (1999) demonstrated that ammonia, produced by *M. anisopliae*, could alter ambient pH and suggested that ammonia production may be responsible for manipulating micro-environmental conditions required for optimising infection, possibly by disturbing the host immune system.

Once the insect exoskeleton has been penetrated via germ-tube or appressorium, the fungus enters the haemocoel and continues its filamentous development (Yendol & Paschke, 1965) or grows vegetatively as yeast-like structures (blastospores), as hyphal bodies (Charnley, 1984), or as protoplasts (Entomophthorales spp.) (Hajek, 1997). These growth forms cannot survive outside of the host and are thought to provide the pathogen with an increased surface area for acquisition of nutrients as they readily disperse and colonize the haemocoel through circulation in the hemolymph, and possible invasion and dissipation of the host immune response (Hajek, 1997). However, many entomopathogenic fungi overcome their host before extensive invasion of organs takes place. In this case, toxins are presumed to be responsible for host mortality. For example, *M. anisopliae* produces a number of destruxins (DTX) including desmethyl destruxins A, B, D and C; destruxins E, E1, E2, A1, A2 A3, B1, B2, C2, D1, D2, chlorhydrid, and other related destruxins (Suzuki *et al.*, 1970; Pais *et al.*, 1981; Gupta *et al.*, 1989; Wahlman & Davidson, 1993; Amiri-Bisheli *et al.*, 2000; Strasser *et al.*, 2000) at levels toxic to many insects (Amiri *et al.*, 1999; Kershaw *et al.*, 1999). The titre of DTX production seems to be correlated with differential virulence against some insects (Kershaw *et al.*, 1999). This kind of metabolism improves the fungi virulence against the insects in such a way that it can avoid the host response and so grows faster into the haemocoel (Hajek & St. Leger, 1994). Although it is evident that destruxins (DTX) are determinants of virulence in *M. anisopliae*, the precise role(s) of these cyclic depsipeptides is difficult to assess (Vey *et al.*, 2001).

Not all fungal cells that successfully penetrate insect cuticle grow and reproduce. Hosts mount humoral and cellular defence responses in the form of melanin or melanin-protein complexes, phagocytosis, nodulation or encapsulation (Butt *et al.,* 1988; St Leger *et al.*, 1988; Butt *et al.*, 1996; Bidochka *et al.*, 1997; Bidochka & Hajek, 1998). In a few cases, behavioural alternations of hosts can cure fungal infection (Hajek *et al.,* 1990; Carruthers *et al.*, 1992).

1.6 AIMS AND OBJECTIVES

The aim of this project was to elucidate the underlying mechanisms of attenuation in the entomopathogen *Metarhizium anisopliae*.

Particular attention will focus on the production of known virulence determinants such as the subtilisin Pr1 and putative determinants such as hydrophobin. Subtilisin and hydrophobins play major roles in cuticle degradation and spore adhesion, respectively. Failure to adhere is a key feature of attenuated strains. At present, no links have been made with attenuation and changes in the production or hydrolytic properties of Pr1. Starvation (derepression) and presence of insect cuticle (induction) can both induce Pr1 and hydrophobin. However, simple sugars and amino acids, if in excess, can repress Pr1 production. These observations suggest that the fungus has "sensing" systems that monitor exogenous and endogenous nutrient availability.

The specific objectives of this project are:

1. To compare Prl production by attenuated and virulent sub-cultures of *M. anisopliae* isolates.
2. To compare the adhesion properties of conidia of attenuated and virulent cultures of *M. anisopliae* and to identify the factors (e.g. mucilage, hydrophobins) which may account for poor adhesion to insect cuticle.
3. To compare the endogenous reserves of attenuated and non-attenuated conidia of *M. anisopliae* as well as starved conidia of the fungus and to conduct bioassays to see how the inoculum from these cultures differ in virulence.

CHAPTER 2

The effects of nutrition on morphological, physiological and pathogenic state of the entomopathogenic fungus, *Metarhizium anisopliae*

2.1 INTRODUCTION

Aphids are considered to be one of the most important pests of UK crops causing damage directly by feeding and indirectly by transmitting plant virus diseases such as barley yellow dwarf virus in cereals, virus yellows in beet crops and leaf roll virus and virus Y in potato crops (Kennedy, 1962; Eskanderi *et al.,* 1979). Furthermore, the sugar-rich honeydew secreted by aphids encourages the growth of saprophytic and pathogenic fungi on crop plants reducing both yield and quality (Bergstrom *et al.,* 1982). Since most aphid species are viviparous and reproduce parthogenetically they can reach pest proportions very quickly.

Pollen beetles, *Meligethes* spp., are major pests of brassica crops throughout Europe, causing economic damage particularly to spring-sown oilseed rape (Winfield, 1992). Both larvae and adult beetles feed on flower buds resulting in pod loss and appearance of blind stalks. At high beetle densities (>10 beetles per plant) plants fail to produce seed of sufficient quantity and quality leading to significant yield losses.

The frequent and extensive use of chemical insecticides has resulted in certain insect species developing widespread resistance to a range of pesticides (Metcalf, 1989). For example, the peach potato aphid (*Myzus persicae*) has developed resistance to a number of carbamate, pyrethroid and organophosphate based insecticides (Foster *et al.*, 1998). The devastating implication of pesticide resistance developing in insect populations, combined with the increased global interest to reduce the input of harmful pesticides, has encouraged research into the development of environmentally benign strategies for pest control including the use of entomogenous fungi (Butt *et al.*, 2001b).

Several isolates of the entomogenous fungus *Metarhizium anisopliae* have been shown to be pathogenic to several cruciferous pests including cabbage stem flea beetles (*Psylliodes crysocephala*), mustard beetles (*Phaedon cochleareae*) and pollen beetles (*Meligethes aeneus*) (Butt *et al.*, 1992; 1994; 1998). The same isolates have also been shown to be pathogenic to the aphid species *Myzus persicae* and *Lipaphis erysimi* (Butt *et al.*, 1995) but harmless to honey bees (*Aphis mellifera)* (Butt *et al.*, 1994).

One of the main requirements for commercially available biocontrol agents is that they can be readily produced in large quantities. However, the continual production of inoculum on artificial culture media may lead to instability and attenuation of virulence of the organism. This phenomenon has been reported by several workers (Fargues & Roberts, 1983; Morrow *et al.*, 1989; Butt & Goettel, 2000) but nothing is known about the underlying mechanisms of attenuation. Virulence is restored by passaging attenuated inocula through a suitable host (Fargues & Roberts, 1983; Morrow *et al.*, 1989; Perenova, 1994).

The aim of this study was to determine the effect of culture media on: (i) the germination of conidia and mycelial growth of *Metarhizium anisopliae* (ii) virulence of *M. anisopliae* against *Myzus persicae* and *Meligethes aeneus* (iii) surface carbohydrates of *M. anisopliae* conidia (iv) accumulation of endogenous reserves in conidia of *M. anisopliae* and (v) biochemistry of *M. anisopliae* conidia.

Better understanding of how nutritional conditions affect virulence of entomogenous pathogen *M. anisopliae* could help to explain underlying mechanisms of attenuation.

2.2 MATERIALS AND METHODS

2.2.1 Collection and rearing of *M. aeneus* and *M. persicae*

M. persicae was reared on plants of Chinese cabbage (var. TipTop) at room temperature and under natural day light conditions with supplementary fluorescent lighting ("Arcadia", Jerrard Bros plc, UK) until required for experimentation. Adult *M. aeneus* were collected from flowers of a crop of winter oilseed rape (var. Apex) grown at Harper Adams University College prior to experimentation.

2.2.2 Maintenance and storage of *M. anisopliae*

Three strains of *M. anisopliae* were used in this study; two (V245 and V234) were isolated from Finnish soils and the third (V208) from an orthopteran host in Brazil. Long-term storage of each isolate was achieved by freeze-drying conidia. When required, conidia were re-hydrated by suspending in a small volume of sterile water, placed on Sabouraud Dextrose Agar (SDA, Lab M, UK) and incubated at 23 ± 1°C in the dark for 14 days. Following incubation, conidia were scraped from sporulating colonies and suspended in 0.03% Tween 80 solution (BDH). The resulting conidial suspension was then filtered three times through glass wool (Pyrex) in order to remove hyphal fragments.

2.2.3 Effect of artificial media on the germination, growth and sporulation of *M. anisopliae in vitro*

Four different agar media, varying in nutritional substrates were prepared: SDA (15 g Agar No.1, 10 g mycological peptone and 40 g glucose per litre sterile distilled water),

SDA modified with KCl (55 g per litre) to raise the osmolarity of the medium (SDAM), Yeast Extract Agar (YEA) (15 g Agar No.1 and 10 g yeast extract per litre of water) and Minimal Medium (15 g Agar No.1, 0.3 g K_2HPO_4, 0.3 g $MgSO_4.7H_2O$, 0.15 g NaCl, 0.3 g $CaCl_2.6H_2O$ (saturated), 0.008 g $MnSO_4.4H_2O$, 0.002 g $CuSO_4.5H_2O$ and 0.02 g $FeSO_4.7H_2O$ per litre of water). Each medium was either amended (+) or unamended (-) with 200 mg l^{-1} of aphid (*M. persicae*) homogenate. The pH of each medium was adjusted to a value of 6.5 before being autoclaved at 121 °C and 103.4 Kpa for 20 min. When cool, each medium was dispensed into 9 cm diam plastic Petri dishes at a rate of 15 ml per dish. For each of the media tested, 10 µl of a conidial suspension (10^5 conidia ml^{-1} water) of fungal isolate was dispensed onto the centre of 10 replicate dishes. All plates were sealed with parafilm 'M' (American National) and incubated at 23 °C in the dark. After 24 hr incubation, five replicate plates of each treatment were removed and the incidence of germinated conidia recorded for 500 conidia (100 conidia per replicate). A spore was considered to have germinated if it had formed a germ-tube that was as long as the spore width (Manners, 1966). The remaining five replicates were examined every three days over the following 15 days when mycelium colony diameters were measured. Conidia production on each media tested was determined by taking randomly three plugs from each of the replicated (five times) plates of three-week-old sporulating cultures using a No. 3 cork borer (6 mm in diameter). Each plug was then placed into 1.9 ml micro centrifuge tube containing 1 ml 0.03% Tween 80. The tubes were vigorously shaken for 30 min (Friffin Ltd, UK) and then filtered through four layers of cheesecloth. Conidial concentrations were determined using the haemocytometer (Webber Scientific International Ltd, UK) and expressed as number of conidia per millilitre per plug.

2.2.4 Effect of artificial media on virulence of *M. anisopliae* against *M. persicae*

Sporulating cultures of *M. anisopliae* V208, V234 and V245 grown on each of the culture media tested were used to prepare separate conidial suspensions ($1.6x10^7$ conidia ml^{-1}) using the procedure described above. For each of the prepared conidial suspensions, 10 adult aphids were inoculated by immersing them for 10 seconds in 5 ml of suspension. Excess conidial suspension was removed by placing inoculated aphids onto filter paper in a Buchner funnel. Ten aphids were then transferred to a leaf disc (50 mm diam) of Chinese cabbage placed on 20 ml of water agar (WA, Lab M, UK) in a 9 cm diam Petri dish. Controls consisted of aphids immersed in 0.03% Tween 80 only. There were three replicates per treatment. All dishes were incubated at 23 °C ± 1 °C with a 16:8 h (Light: Dark) photoperiod and inspected daily. The number of dead aphids was recorded over a period of 7 days and lethal time (LT_{50}, the time taken to kill 50% of individuals) was extrapolated for each treatment. Cadavers were transferred to Petri dishes lined with moist tissue paper to encourage fungal growth and sporulation in order to confirm that death was due to infection by *M. anisopliae*.

In order to study the germination and germling development of conidia of *M. anisopliae in vivo*, conidial suspensions (10^7 spores ml^{-1}) of each fungal isolate were obtained from sporulating cultures grown on either SDA, SDAM, YEA or MM agar amended with aphid homogenate. These media were chosen since they provided a range of LT_{50} values during the mortality studies outlined above. For each treatment, aphids were inoculated by dispensing 2 µl aliquots of conidial suspension onto the dorsal site of individuals using a Gilson pipetteman. Inoculated aphids were then incubated at 23 °C under a 16 h

photoperiod for 43 hours before being frozen at –20 °C. When required, five aphids were removed from the freezer for each treatment and examined under a fluorescence Leitz

DM-RB photo microscope with MPS 48/52 photoautomat exposure control (Butt *et al.*, 1995). A further five aphids were also taken for each treatment and desiccated over silica gel for over 24 hr in order to remove unbound water before coating each individual with a conductive molecular layer of gold prior to examination under a Cambridge S200 scanning electron microscope (SEM). For both fluorescence and SEM, the total number of germinated and ungerminated conidia found on the inoculated dorsal site of individuals was recorded along with the number of germlings that had produced an appressorium.

2.2.5 Effect of artificial media on virulence *of M. anisopliae* against *M. aeneus*

To determine if cultural conditions altered host specificity, *M. anisopliae* was assayed against adult *M. aeneus*. In these studies, only isolate V245 was used due to its ease in culturing and readiness to sporulate. Separate conidial suspensions (1.6×10^7 conidia ml^{-1} 0.03% (v/v) Tween 80) were prepared from sporulating cultures grown on each of the media tested. Ten adult pollen beetles were inoculated by immersion in 10 ml of conidial suspension for 10 seconds before being transferred to a Buchner funnel to remove excess suspension. Controls consisted of beetles treated with 0.03% Tween 80 only. Inoculated beetles were then placed in groups of 10 in ventilated perspex boxes (5.5 x 11.5 x 17.5) lined with moist tissue paper. Three freshly cut inflorescences of oilseed rape were placed in each box as food. Three replicate boxes were prepared for each treatment. Over the 10 day incubation period identical to that described for peach potato aphids, pollen beetles were inspected daily and the number of mortalities recorded. As with aphids, dead pollen

beetles were removed and placed in a Petri dish lined with moist filter paper to encourage external sporulation.

Studies on conidial germination and germling development of isolate V245 were also carried out *in vivo* using pollen beetles. Separate conidial suspensions (10^7 conidia ml^{-1} 0.03% (v/v) Tween 80) of the pathogen were prepared from colonies growing on each of the eight culture media and used to inoculate the dorsal site of adult beetles as described above. Following incubation at 23 °C and 16 h photoperiod for 48 hr, beetles were frozen before being prepared for fluorescence and SEM examination. Total conidia, percentage conidial germination and percentage germinated conidia which had developed appressoria was recorded for each treatment.

2.2.6 Effect of media on the carbohydrate residues at the conidial surface

Good adhesion is an attribute of virulent strains of entomogenous fungi (Al-Aidroos & Roberts, 1978). Fluorescence microscopy techniques were used to determine if culture media affected the carbohydrate composition at the surface of the conidial cell wall and subsequently spore adhesion. The carbohydrate probes used were: calcofluor, a vital stain that binds to ß-glucans, and the FITC-labelled lectins concanavalin A (Con A) specific for α-D-mannose and α–D-glucose, wheat germ agglutinin (WGA) specific for N-acetyl-D-glucosamine and *Ricinus communis* agglutinin (RCA) specific for β–D-galactose and N-acetyl-D-galactosamine (Butt, 1997).

Sporulating cultures of all three isolates of *M. anisopliae* grown on each of the culture media were used to prepare separate conidial suspensions (10^7 conidia ml^{-1}). For each

conidial suspension, 10 ml were centrifuged for 15 min at 2000 x g (Model TJ-6 Centrifuge, Beckman®, USA), washed in 10 ml of 0.03% Tween 80 three times and re-suspended in 10 ml of 0.01% aqueous Fluorescent brightener 28 (Calcofluor White M2R, Sigma). The suspension was then incubated overnight at room temperature in the dark. Following incubation, the stained conidia were washed three times in sterile distilled

water and re-suspended in 10 ml of 0.03% Tween 80 before examination under a fluorescence microscope. Since the brightness of fluorescence is difficult to estimate objectively and depends partly on the type and quality of the microscope used, an LS 30 luminescence spectrometer (Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire, England) operating at an excitation wavelength of 350 nm and an emission wavelength of 435 nm was used, which allowed the measurement of fluorescence intensity more accurately. Five replicates were assessed for each treatment.

Conidia-lectin binding assays were performed for each isolate and for each culture medium as follows: 25 µl of 10 mM PBS solution (PBS with 0.1% sodium azide, pH 7.2 (Sigma)), 70 µl of conidial suspension and 5 µl of lectin (1mg ml^{-1}) in PBS solution were placed in a 1.9 ml Eppendorf tube. The content of the tube was mixed gently then wrapped in foil and incubated for 30 min at room temperature in the dark. Conidia were then washed three times with PBS. Carbohydrate specificity controls were performed by comparing the fluorescence intensity between conidia incubated for 30 min in the lectin solution and in the lectin solution with the appropriate inhibitory carbohydrates (Sigma) after both solutions had been incubated for 90 min at 37° C. The inhibitory carbohydrates were used at final concentrations of 200 mM.

Lectin binding to conidia was manifested as wall fluorescence and categorised according to its intensity: 0 - no observed fluorescence, 1 - weak (photoautomat exposure control indicated >30 min), 2 - moderate (photoautomat exposure control indicated 1–30 min) and 3 - strong (photoautomat exposure control indicated <1 min). Fluorescence indices (total fluorescence score divided by total number of conidia) were divided by three to give a value between 0 - 1 and analysed using analysis of variance. One hundred conidia were examined in each replicate with five replicates for each treatment.

2.2.7 Polyols and trehalose analysis

Aerial conidia of all three isolates were harvested from each of the culture media tested and from aphid cadavers by gently scraping the surface of a culture plate into Water G Chromasolv® (Riedel-deHaën, Laborchemikalion Gmb & Co., Germany) containing 0.03% Tween 80. Suspended conidia were filtered three times through glass wool to remove any hyphal fragments and washed in Water G three times by centrifugation at 3800 r.p.m for 15 min. The resultant spore pellets were frozen at –80 °C before being lyophilised in an Edwards Modulyo freeze dryer (Severn Scientific Limited, UK). Fifteen mg of freeze-dried conidia from each culture media and for each of the three isolates were homogenised in 1 ml Water G for 30 sec on ice using micropestels fitted to a Black & Decker drill (BL500R, Ø15mm). 1.9 ml micro centrifuge tubes containing conidial homogenate were immersed in a boiling water bath for 5 min. The crude extracts were collected by centrifugation of conidial homogenates for 20 min at 13 000 r.p.m (Biofuge 13, Heraeus Instruments Ltd, UK). The resultant supernatants were passed through VectraSpin Micro tube filters (5K

MWCO) (Whatman International Ltd, UK) before being assessed for polyols (mannitol, arabitol, erythritol) and trehalose content.

For high performance liquid chromotography (HPLC), a HP 1100 system (Hewlett Packard) combined with HP 1100 diode array detector and Sugar-Pak™1 (6.5 x 300.0 mm) column from Waters (UK) was used. The best separation of polyols and trehalose was achieved by using mobile phase of Ca^{+}-EDTA in Water G Chromasolv® and under constant flow rate of 0.5 ml min^{-1}. The column was heated (70° C) by HP 1100 column thermostat. A standard solution was made up of highest available grades of polyols (mannitol, arabitol, erythritol) and trehalose (Sigma) each at 100 mg ml^{-1}. A serial dilution for each compound was carried out to give a range of concentrations (from 0.1 to 100 µg ml^{-1}) so that each injection of conidial extract was preceded and succeeded by injections of standards that could be detected at the same sensitivity. 50 µl of standard solution was used to calibrate the HPLC. The same injection volume of 50 µl of conidial extracts was used for comparisons. The compounds were detected and identified by their retention time. The polyols and trehalose content of conidia were calculated using the mean values of peaks from traces of standards that came immediately before and after sample traces (Hallsworth & Magan, 1997).

2.2.8 Total Carbon, Sulphur and Nitrogen analysis

Aerial conidia were harvested from each of the culture media tested and from the aphid cadavers by gently scraping the surface of a culture plate into Water G Chromasolv® (Riedel-deHaën, Laborchemikalion Gmb & Co., Germany) containing 0.03% Tween 80. Suspended conidia were filtered three times through glass wool to

remove any hyphal fragments and washed in Water G three times by centrifugation at 3800 r.p.m for 15 min. The resultant spore pellets were frozen at –80 °C before being lyophilised in an Edwards Modulyo freeze dryer (Severn Scientific Limited, UK). Freeze-dried conidia were then assessed for carbon, sulphur and nitrogen content using a SC-144DR Carbon/Sulfur Analyser and a FP-528 Protein/Nitrogen Analyser, respectively (Leco Corporation, USA).

2.2.9 Analytical isoelectric focusing (IEF)

Crude conidial extracts prepared for polyol and trehalose analysis were also used for analytical IEF. The total protein content of these extracts was quantified using Bio-Rad Protein microplate assay (Kit II, Bio-Rad Laboratories Ltd, UK) (Bradford method), which is based on the differential colour change of a dye in response to various concentrations of protein (Bradford, 1976). A standard curve was determined each time the assay was performed. Protein concentration of each sample was calculated from the absorbance readings at optical density (OD_{560}) only if the correlation coefficient of the standard curve was ≥ 0.985.

Analytical IEF was performed according to Garfin (1990). Samples (20 µl) of conidial extracts were applied alongside pI-markers onto paper strips to a 1 mm thick precasted gel (Ampholine® PAGplate; Pharmacia Biotech) and fractionated using Pharmacia LKB Multiphor II (Pharmacia) system with ampholine pH range of 4 to 6.5.

Cathode and anode electrode strips were soaked in 1.0 M NaOH and 1.0 M H_3PO_4, respectively. The gel was pre-focused for 30 minutes with 800 V at 4 °C, followed by electrophoresis for 1.5 hr under the same conditions. After electrophoresis, protein bands on these gels were detected using either Coomassie stain (0.5% Coomassie brilliant blue R250 in 25% ethanol and 10% acetic acid at 60° C for 30 min) or periodic acid with

Schiff's reagent (PAS) staining for glycoproteins (based on method of Dubray & Bezard, 1982). Briefly, after separation of the proteins, the gel was soaked in a solution containing 25% isopropyl alcohol and 10% acetic acid overnight at room temperature. Rinsed with distilled water the gel was soaked in 7.5% acetic acid for 30 min and then placed in 0.4% aqueous periodic acid for 1 hour at 4 °C. The solution was poured off and the gel immediately placed in Schiff's reagent without rinsing and refrigerated for at least 1 hour. Stained gel was destained in several changes of 7.5% acetic acid at room temperature until the solution was no longer pink.

2.2.10 SDS-Polyacrylamide gel electrophoresis

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) protein separation was performed according to the method of Laemmli (1970) using 1mm thick gel (12.5% resolving and 5% stacking gel) and Mini-PROTEAN II electrophoresis system (Bio-Rad, UK). Protein patterned gels were stained with 0.5% Coomassie blue (see section 2.2.9) and subsequently destained with several changes of 50% ethanol and 7% acetic acid solution.

2.3 RESULTS

2.3.1 Effect of artificial media on the germination, growth and sporulation of *M. anisopliae in vitro*

The germination rate of *M. anisopliae* conidia was dependent on the substrate and isolate assayed (Fig. 2.1a-c). On all substrates, the speed and percentage germination of conidia was higher for V234 followed by V245 then V208. The maximum germination was observed on SDA media for all of the isolates tested. Spore germination was poor or inhibited on SDAM media after 24 h incubation (Plate 2.1a). The *M. persicae* homogenate stimulated conidial germination for almost all the isolate-substrate combinations (Fig. 2.1a-c, Plate 2.1d) with the exception of SDAM (Plate 2.1b) and MM media for V208 (Fig. 2.1b). YEA alone, and in combination with insect homogenate, produced high percentage germination for all isolates reaching 82 and 93% for V234, 74 and 79% for V245, and 56 and 75% for V208, respectively.

The initial nutrient availability also affected the fungal growth ($F_{7,92} = 2164.98$, $P<0.001$), which was faster on rich SDA and significantly slower on KCl-amended media (Fig. 2.2a-c). Addition of aphid homogenate appeared to accelerate growth within most treatments except for SDAM. The growth of all the strains of *M. anisopliae* was sparse in nutrient poor media (MM) but denser in nutrient rich media. The cultural and morphological characteristics of *M. anisopliae* isolates: shape, profile, surfaces and margins of colonies, colouration of aerial and substrate mycelium, colouration of aerial conidia, saltation, formation and excretion of the pigment into the nutrient media and conidium production are shown to depend on the nutrient media (Plate 2.2a-c).

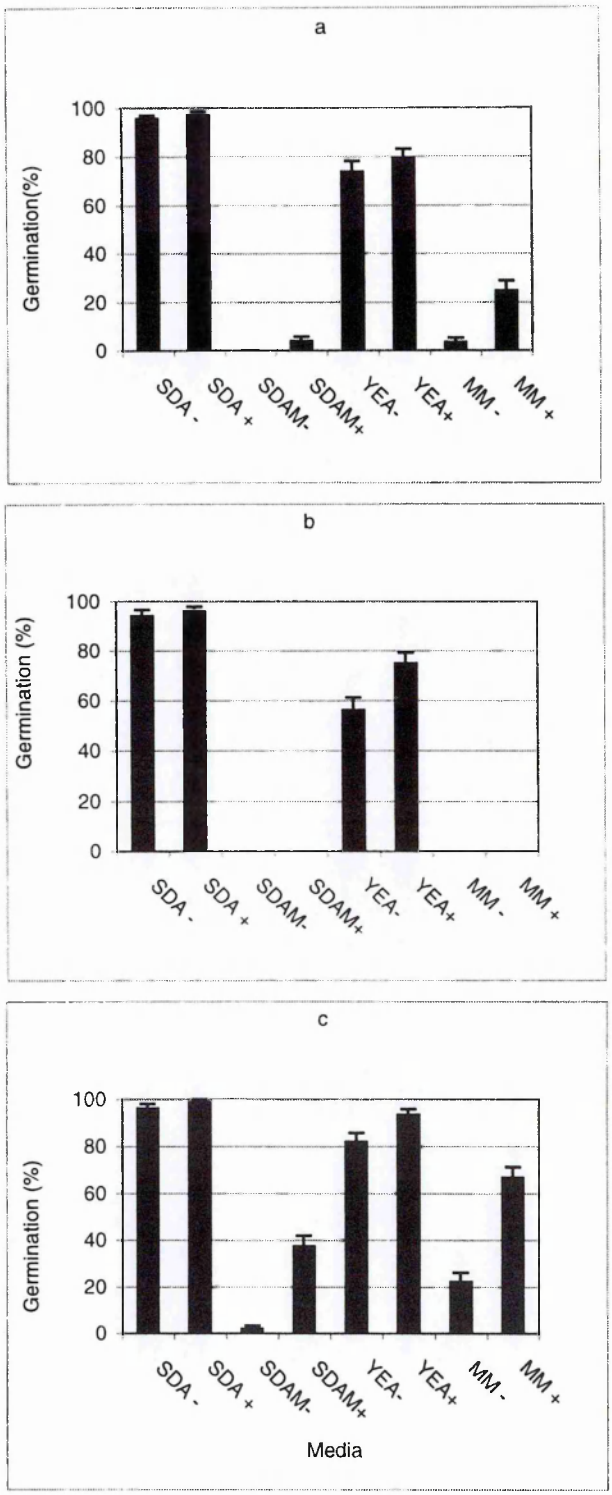
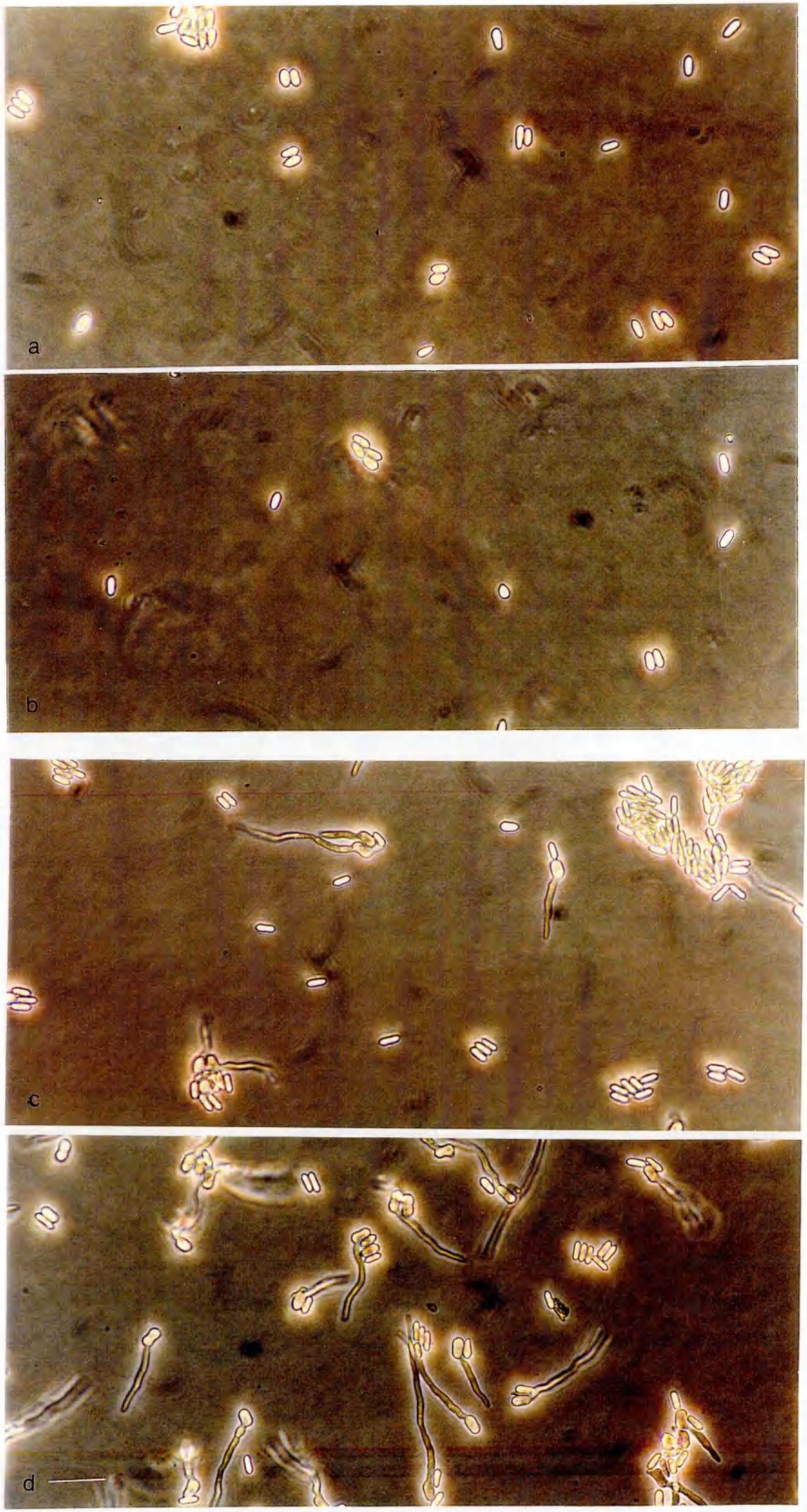


Figure 2.1a-c. Effect of culture media on the germination of three isolates of *Metarhizium anisopliae*; V245 (a), V208 (b) and V234 (c) following 24 h incubation at 23 °C. SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. The bars represent of standard errors.

Plate 2.1a-d. Effect of culture media on germination behaviour of *Metarhizium anisopliae,*x 400; V208 on SDAM- (a), V208 on SDAM+ (b), V234 on MM- (c) and V234 on MM+ (d) following 24 h incubation at 23 °C. SDAM = SDA amended with 55 g l^{-1} KCl, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. Bar = 20 μm.


a
b
c
d

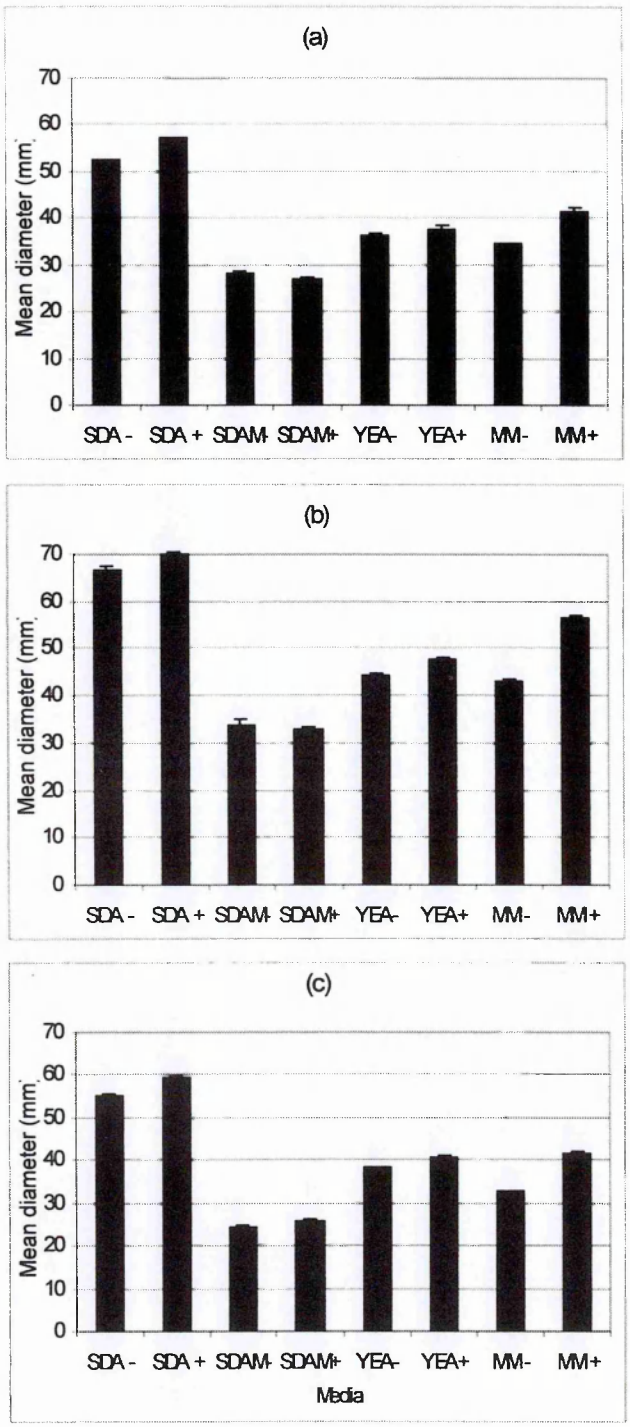


Figure 2.2a-c. Effect of culture media on colony diameter of three isolates of *Metarhizium anisopliae*; V245 (a), V208 (b) and V234 (c) following incubation for 15 d at 23 °C. SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. The bars represent standard errors.

Conidial production was isolate dependent ($F_{2,46}$ = 100.5, P < 0.001) and significantly influenced by nutritional conditions ($F_{7,46}$ = 2179.72, P < 0.001). The highest conidial yield, ranging from 5.5 X 10^7 to 1.0 X 10^8 conidia ml^{-1} $plug^{-1}$, was obtained from cultures grown on nutrient-rich (SDA-) media (Table 2.1). Nutrient-deficient medium (MM-) although having a beneficial impact on the onset of initial conidiation, was shown to significantly reduce total conidia concentrations for all three isolates tested. Addition of aphid homogenate had no effect or considerably reduced fungal sporulation depending on the isolate evaluated (Table 2.1).

2.3.2 Effect of culture media on virulence of *M. anisopliae*

All the isolates were highly pathogenic for *M. persicae* causing 90-100% mortalities within 7 days and for *M. aeneus* causing 70-100% death within 10 days regardless of the substrates the inoculum was produced on (Tables 2.2 and 2.4). Control mortality ranged between 0 and 3% for all treatments.

The LT_{50} values of each strain infecting aphids differed significantly ($F_{2,694}$ = 39.15, P<0.001). Media also significantly influenced fungal virulence ($F_{7,694}$ = 31.41, P<0.001) and the interaction was also significant ($F_{14,694}$ = 4.04, P<0.001). The greatest LT_{50} values observed for V245, V208 and V234 were 5.5 on SDA+, 5.7 on SDA- and 5.4 on SDA+, respectively (Table 2.2). Conidia produced on nutrient-poor media (MM-, MM+)

Plate 2.2a-c. Effect of culture media on cultural and morphological characteristics of three isolates of *Metarhizium anisopliae*; V245 (a), V208 (b) and V234 (c) following incubation for 15 d at 23 °C. 1. SDA = Sabouraud Dextrose Agar, 2. SDAM = SDA amended with 55 g l^{-1} KCl, 3. YEA = Yeast Extract Agar, 4. MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.

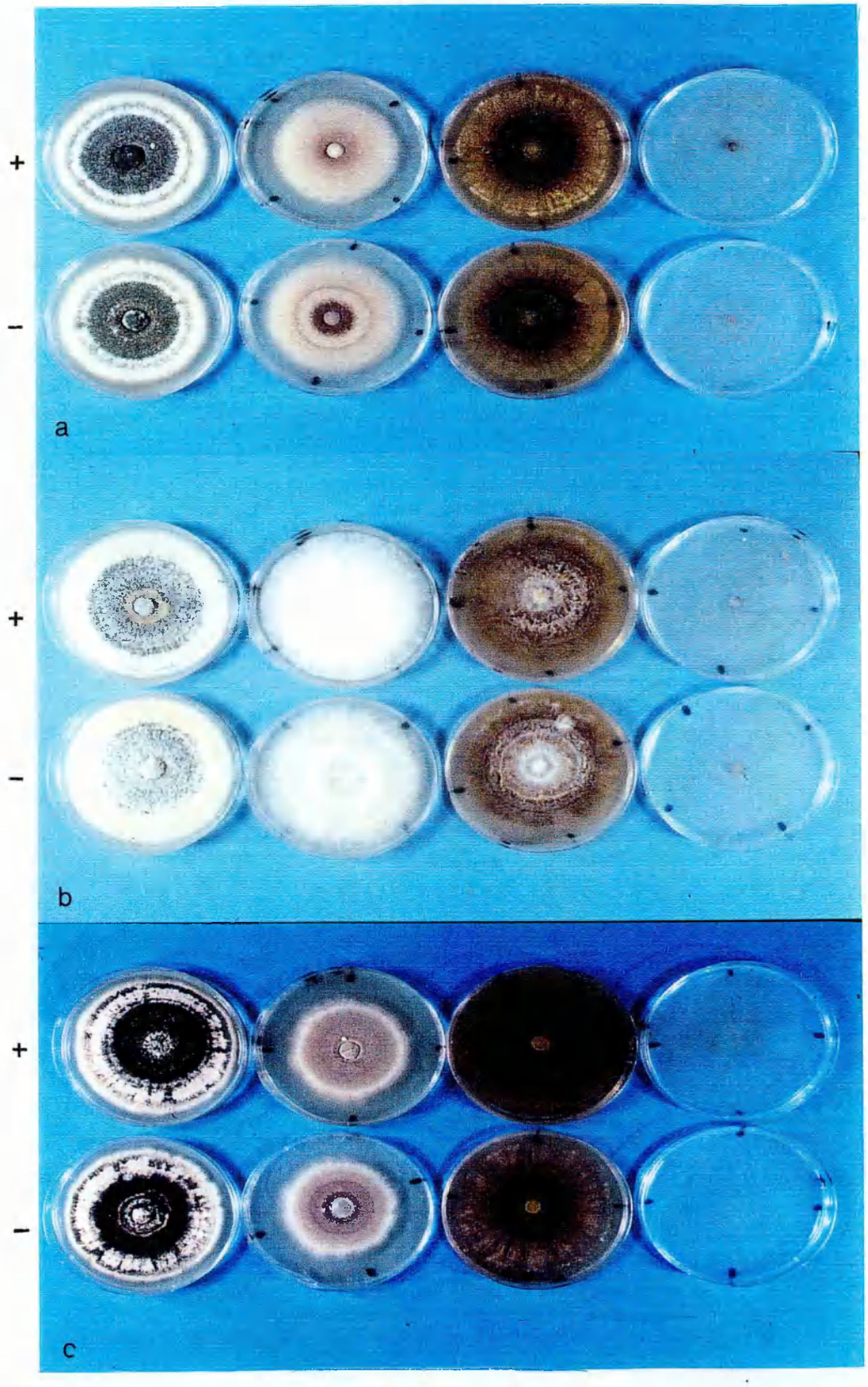

+
–
a
+
–
b
+
–
c
SDA
SDAM
YEA
MM

Table 2.1. Conidia production by three isolates of *Metarhizium anisopliae* cultured on different culture media 21 days after inoculation.

Media[a]	10^6 conidia ml^{-1}[b]		
	Isolate		
	V245	V208	V234
SDA-	103 .0	54.9	85.0
SDA+	70.7	56.6	101.3
SDAM-	4.7	17.5	23.3
SDAM+	1.4	10.2	8.7
YEA-	16.6	12.4	6.2
YEA+	13.4	11.4	8.7
MM-	2.3	4.4	2.0
MM+	3.4	3.2	3.0

LSD[c] (P< 0.001)	
Isolate	1.2
Media	2.0
Isol*med	3.5

[a] SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.
[b]Each value represents the combined means of five replicate Petri dishes.
[c]LSD=least significant difference.

Table 2.2. Percentage mortality and lethal time (LT_{50}) obtained for three isolates of *Metarhizium anisopliae* cultured on different growth media against *Myzus persicae* following 7 days incubation at 23 °C.

Medium[a]	Isolate					
	V 245		V208		V234	
	Aphid mortality[b] (%)	LT_{50}[c] (days)	Aphid mortality (%)	LT_{50} (days)	Aphid mortality (%)	LT_{50} (days)
SDA -	100	5.4 (0.84)	90	5.7 (0.79)	100	5.2 (0.84)
SDA +	100	5.5(1.16)	100	4.9 (1.23)	93	5.4 (0.85)
SDAM -	100	4.9 (1.08)	100	3.8 (0.84)	100	4.3 (0.55)
SDA M +	100	5.0 (0.41)	100	3.7 (0.60)	100	4.9 (0.91)
YEA -	100	5.0 (0.59)	100	4.5 (0.65)	100	5.1 (0.64)
YEA +	100	5.4 (0.57)	100	4.2 (1.11)	100	5.0 (0.67)
MM -	100	4.5 (0.38)	100	3.2 (0.49)	100	4.6 (0.81)
MM +	100	3.8 (0.53)	100	3.5 (1.03)	100	4.0 (0.68)
LSD						
Isolate	0.1716 (P<0.001)					
Medium	0.2802 (P<0.001)					
Isol*Med	0.4854 (P<0.001)					

[a] SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.

[b] Total mortality 7 days post-inoculation.

[c] LT_{50} = time in days when 50% of *M. persicae* individuals were dead. Numbers in parentheses are standard errors.

had significantly lower LT_{50} values for all isolates and gave values of 3.8 (V245), 3.3 (V208) and 4.0 (V234) days. Similar ($F_{7,230} = 19.02$, $P < 0.001$) results were obtained for V245 against pollen beetle, the highest and lowest values being 7.7 days (SDA-) and 3.8 days (SDAM+), respectively (Table 2.4).

2.3.3 Adhesion and germination of *M. anisopliae* conidia on insect cuticles

The germination pattern of *M. anisopliae* on aphid and pollen beetle cuticles is summarised in Tables 2.3 and 2.4. The number of conidia adhering to the live aphids was not significantly different between isolates ($F_{2,44} = 3.18$, $P>0.05$) but significant differences were noted between the culture media treatments ($F_{3,44} = 17.59$, $P<0.001$) with no interaction ($F_{6,44} = 1.39$, $P>0.05$). Many V245 conidia adhered to the surface of aphid (>100) and beetle (84) cuticle if grown on media containing KCl. In contrast, 50% fewer conidia adhered to aphid cuticle, irrespective of strain, if grown on MM. Although the percentage spore germination 43 hr post-inoculation was not significantly different ($F_{2,44} = 1.65$, $P>0.05$) among the strains, it was far more dependent on the substrate used ($F_{3,44} = 54.05$, $P<0.001$). Statistical analysis revealed a significant effect of culture medium on spore germination of the respective strain ($F_{6,44} = 4.42$, $P=0.001$). For example, more than 90% of V234 conidia produced on MM substrate had germinated compared to only 16% when conidia were produced on the media amended with KCl. Similar results were obtained when conidia were applied to pollen beetle cuticles; conidia obtained from nitrogen-limited substrates germinated 1.8 times faster than conidia from SDAM ($F_{7,28} = 12.87$, $P<0.001$).

Table 2.3. Percentage of germinated conidia and of germlings which had produced appressoria on the cuticle of *Myzus persicae* individuals of three isolates of *Metarhizium anisopliae* grown on different media 43 hours after inoculation.

Medium[a]	Isolate								
	V 245			V208			V234		
	Conidia[b] (n)	Germinated [c] (%)	Appressoria[d] (%)	Conidia (n)	Germinated (%)	Appressoria (%)	Conidia (n)	Germinated (%)	Appressoria (%)
SDA -	70.2	71.2	44.0 (48.2)	71.2	79.2	17.6 (9.2)	57.8	68.0	38.4 (39.0)
SDAM -	100.0	34.6	23.0 (19.2)	90.2	51.2	24.4 (17.6)	100.0	16.0	14.1 (9.8)
YEA -	83.6	72.4	29.1 (24.6)	82.8	59.2	22.8 (16.2)	48.8	74.6	39.9 (41.4)
MM +	52.2	84.2	36.2 (35.2)	49.2	87.0	61.3 (75.8)	43.2	90.6	55.1(67.0)
LSD									
Isolate	11.79 (P=0.051)	7.59 (P=0.203)	5.44 (P=0.138)						
Medium	13.61 (P<0.001)	8.77 (P<0.001)	6.28 (P<0.001)						
Isol*Med	23.58 (P=0.239)	15.18 (P=0.001)	10.87 (P<0.001)						

[a] SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.
[b] Total number of conidia adhered at the site of conidial application.
[c] Percentage of observed conidia which had germinated.
[d] Percentage of germinated conidia which had produced appressoria. Numbers in parentheses are back-transformed means.

Table 2.4. Percentage mortality, lethal time (LT_{50}) and conidial germination behaviour recorded for isolate V245 of *Metarhizium anisopliae* cultured on different growth media on *Meligethes aeneus* (pollen beetle).

Medium[a]	LT_{50}[b] (days)	Mortality[c] (%)	Number of conidia observed[d] (n)	Germinated conidia[e] (%)	Germinated conidia with appressoria[f] (%)
SDA-	7.7 (0.7)	70	22.0	55.9(68.4)	30.8 (27.0)
SDA+	6.1 (0.8)	93	39.4	72.3(90.0)	54.3 (65.8)
SDAM-	4.8 (0.5)	100	24.6	18.6(13.0)	0.0 (0.0)
SDAM+	3.8 (0.4)	100	83.6	38.6(40.0)	27.9 (28.0)
YEA-	5.8 (1.6)	90	39.2	47.9(55.0)	29.7(25.6)
YEA+	6.0 (0.4)	90	31.8	59.3(73.2)	22.2 (15.2)
MM-	4.3 (0.8)	100	52.6	52.2 (61.2)	43.0 (51.8)
MM+	4.3 (0.3)	100	49.6	68.4 (85.0)	67.8 (85.2)
LSD (P<0.001)	0.83		21.93	13.84	17.77

[a] SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.

[b] LT_{50} = time in days when 50% of *M. aeneus* individuals were dead. Numbers in parentheses are standard errors.

[c] Total mortality 10 days post-inoculation.

[d] Total number of conidia adhered at the site of conidial application.

[e] Percentage of observed conidia which had germinated. Numbers in parentheses are back-transformed means.

[f] Percentage of germinated conidia which produced appressoria. Numbers in parentheses back-transformed means.

The number of germinated conidia which produced appressoria was similar for all isolates ($F_{2,44}$ = 2.07, P>0.05) and reflected the data recorded for spore germination. Depending on culture conditions, the production of appressoria by *M. anisopliae* on aphid cuticle ($F_{3,44}$ = 32.95, P<0.001) varied from 35.2% to 75.8% on nutrient-deficient media with aphid homogenate (MM+) and from 9.2% to 48.2% on nutrient-rich media (SDA-) or from 16.2% to 41.4% on YEA and from 9.8% to 19.2% on SDA amended with KCl. Statistical analysis also revealed a significant interaction between isolate and media ($F_{7,44}$ = 10.07, P<0.001). The presence of insect homogenate in the media consistently increased appressoria production for all three fungal isolates on the surfaces of both aphids and pollen beetles ($F_{7,28}$ = 11.36, P<0.001). For both aphids and pollen beetle, linear regression analysis revealed a significant positive relationship (r^2 = 0.5, P<0.01 and r^2 = 0.71, P<0.01, respectively) between the incidence of conidia which had germinated on insect cuticle and the incidence of appressorial formation. Similar analysis also revealed a significant positive relationship between the number of conidia which had successfully adhered to the cuticle of pollen beetles and LT_{50} value (r^2 = 0.54, P<0.01).

For most media, appressoria, which were produced within 43 hr, were observed to develop directly from conidia (Plate 2.3a) or at the end of relatively short germ-tubes (Plate 2.3c), or directly from conidia as a combination of an appressorium and a penetration peg (Plate 2.3b). However, where conidia were produced on MM medium, the development of long germ-tubes that grew over the surface of the host cuticle producing complex (Plate 2.3d) or a single large appressoria (Plate 2.4c-d) was observed. The morphology and dimensions of appressoria were similar for all isolates.

Plate 2.3a-d. Germination behaviour of *Metarhizium anisopliae* isolate V245 on aphid cuticle following incubation for 43 hr at 23 °C (fluorescence microscopy, x1000). **a.** two germinated conidia, one of which produced a long, narrow appressorium directly from conidium (arrows) (SDA-); **b.** conidia harvested from SDAM- , one of the conidium produced an appressorium and a penetration peg (arrow); **c.** YEA-grown conidium formed an appressorium on the end of relatively short germ-tube; **d.** a complex appressorium formed on the end of a long germ-tube (MM media). SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. Bar = 5 μm.

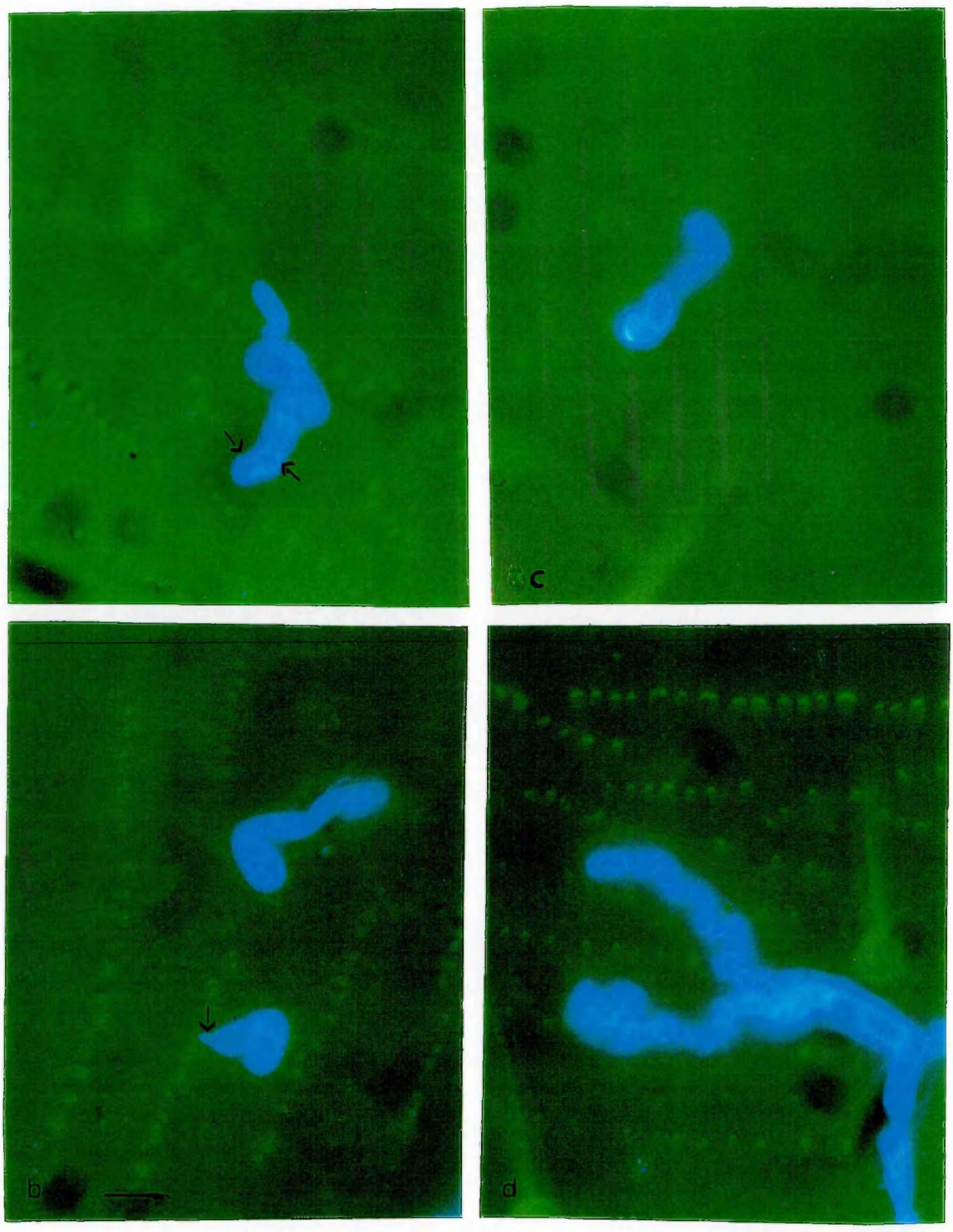
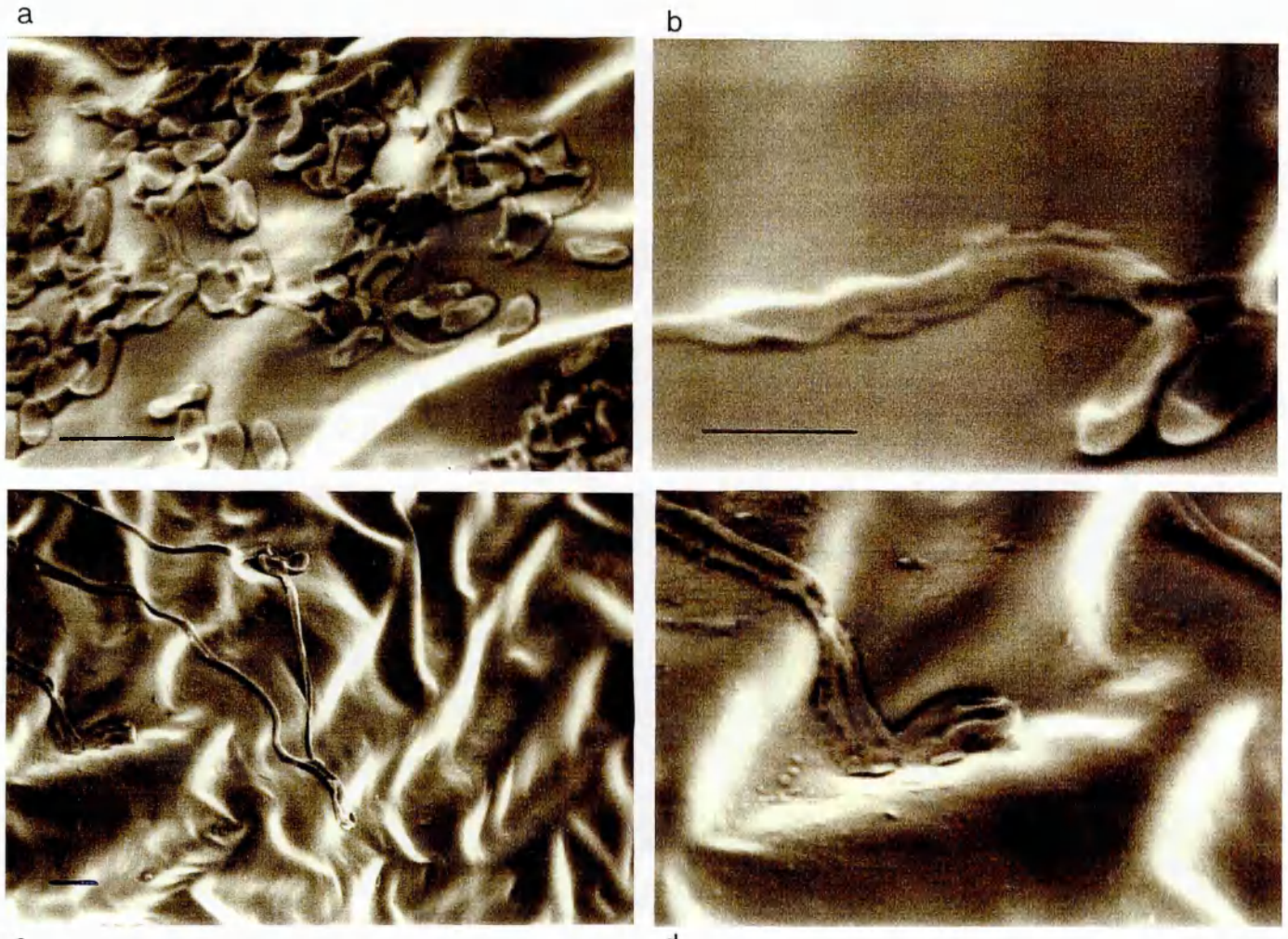

c
b
d

Plate 2.4a-d. Scanning electron micrographs (SEM) of conidia of *Metarhizium anisopliae* isolate V245 on aphid cuticle following incubation for 43 hr at 23 °C. **a.** SDAM-grown conidia. Note those conidia that had germinated produced no appressoria. Bar =10 μm; **b.** same conida as in a. Note mucilage released along the thin germ-tube. Bar = 5 μm; **c.** MM-grown conidia produced large single appressoria at the end of the long germ-tubes. Bar = 10 μm; **d.** same as in c. Note a great amount of mucilage secreted along the germ-tube and around appressorium. Bar = 5 μm. SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.

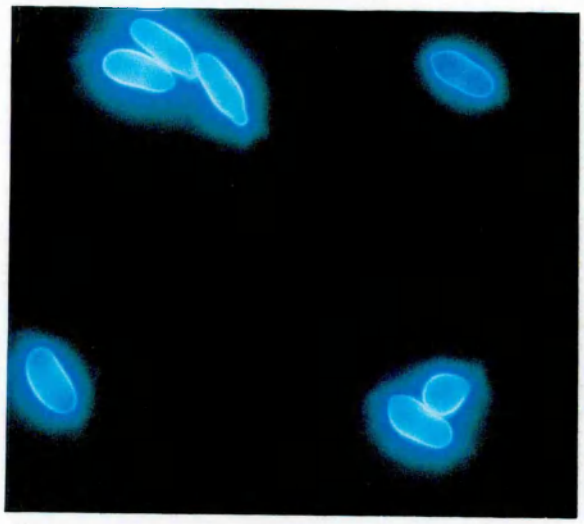

a
b
c
d

2.3.4 Effect of media on the carbohydrate residues at the conidial surface

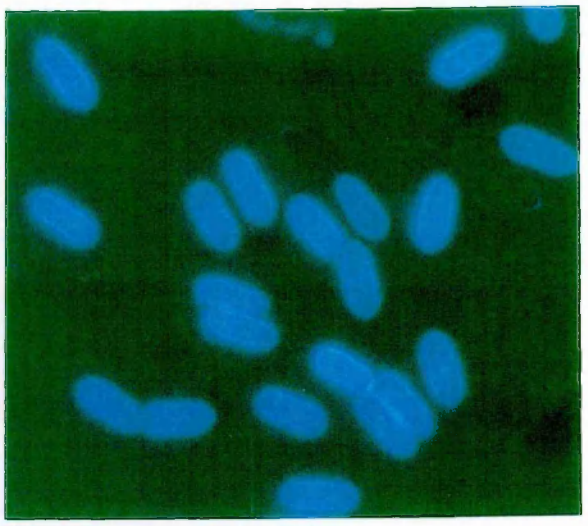
Conidia were found to vary considerably in their pigmentation, size and shape depending on the culture media used. For example, spores produced on SDA and MM media, either with or without aphid homogenate, were cylindrical, dark green in colour and were organised in straight chains (Plate 2.5k), which were difficult to break. Spores from YEA media showed variable pigmentation, ranging from yellow to bright green. Unlike other spores, conidia from SDAM were very pale in colour and lacked chain organisation. Most of the spores were coated in some kind of deposits (Plate 2.5h-i). Also, the size of spores produced on SDAM varied considerably and had a distinct tear drop-like shape (Plate 2.5a, e-i).

Conidia stained with calcofluor fluoresced to different degrees depending on cultural conditions ($F_{7,92} = 174.76$, $P<0.001$) (Plate 2.5a-d, Table 2.5). Conidia of V245 derived from SDAM fluoresced 24 and 4 times more intensely than those derived from MM and SDA, respectively. Statistical analysis of transformed $\log_{10}$ data showed significant differences between isolates ($F_{2,92} = 54.65$, $P<0.001$) that had been grown on the different media and was reflected in their calcofluor-binding capacity (i.e. intensity of fluorescence) ($F_{14,92} = 17.27$, $P<0.001$). When fluorescence intensity was compared with the number of conidia adhering to the insect cuticle, a linear relationship between two variables was observed in the majority of cases. For example, a significant relationship was observed between fluorescence and adhesion for conidia of isolate V245, irrespective of culture media ($r = 0.980$-0.999, $P<0.001$). A similar relationship (i.e. intensity of fluorescence and adhesion of spores to insect cuticle) was observed for V208 isolate

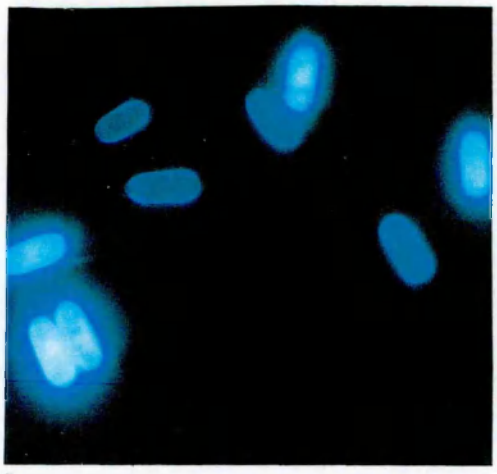
Plate 2.5a-k. Conidia of *Metarhizium anisopliae* isolate V208 stained with calcofluor emit blue fluorescence of different intensity (x1000). **a.** conidia harvested from SDAM-; **b.** conidia harvested from SDA-; **c.** conidia harvested from YEA-; **d.** conidia harvested from MM-. **e-g.** Reactivity of WGA lectin with the cell walls of conidia of *Metarhizium anisopliae,* isolate V208 grown on SDAM. **e.** strong fluorescence; **f.** moderate fluorescence; **g.** weak fluorescence. **h.** reactivity of Con A lectin with the cell wall of a conidium (V208) harvested from SDAM. Note fluorescence of the material deposited to the surface of this conidium (arrow). Bars = 10 μm. **i-k.** SEM of conidia harvested from: **i.** SDAM- and **k.** MM-. Bar = 5 μm. SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.



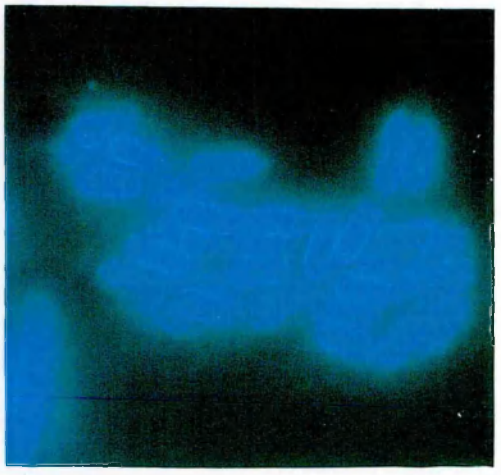
a



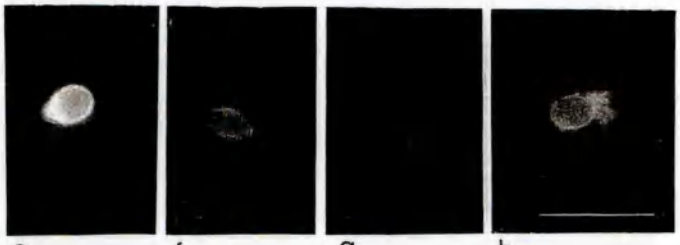
b



c

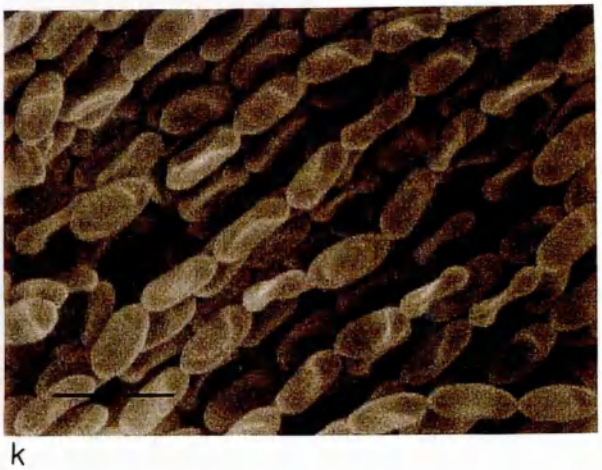


d



e f g h

i



k

Table 2.5. Fluorescence intensity (arbitrary units) of conidia as determined by an LS 30 luminescence spectrometer operating at an excitation wavelength of 350 nm and an emission wavelength of 435 nm for three isolates of *Metarhizium anisopliae* cultured on different growth media (transformed logged base 10 data).

Medium[a]	Isolate		
	V245	V208	V234
SDA-	1.68 (48.0)	1.53 (35.2)	1.67 (48.6)
SDA+	1.75 (56.2)	1.67 (48.8)	1.61 (41.2)
SDAM-	2.32 (208.4)	2.35(224.0)	2.21 (161.2)
SDAM+	2.14 (139.6)	2.26 (181.0)	2.44 (277.2)
YEA-	1.69 (51.0)	1.89 (79.8)	1.66 (46.2)
YEA+	1.73 (54.4)	2.01 (105.0)	1.83 (69.0)
MM-	0.94 (8.8)	1.70 (54.6)	1.35 (23.2)
MM+	1.07 (11.8)	1.85 (74.0)	1.38 (25.2)

LSD
Isolate 0.04 ($P< 0.001$)
Media 0.07 ($P< 0.001$)
Isol*med 0.13 ($P< 0.001$)

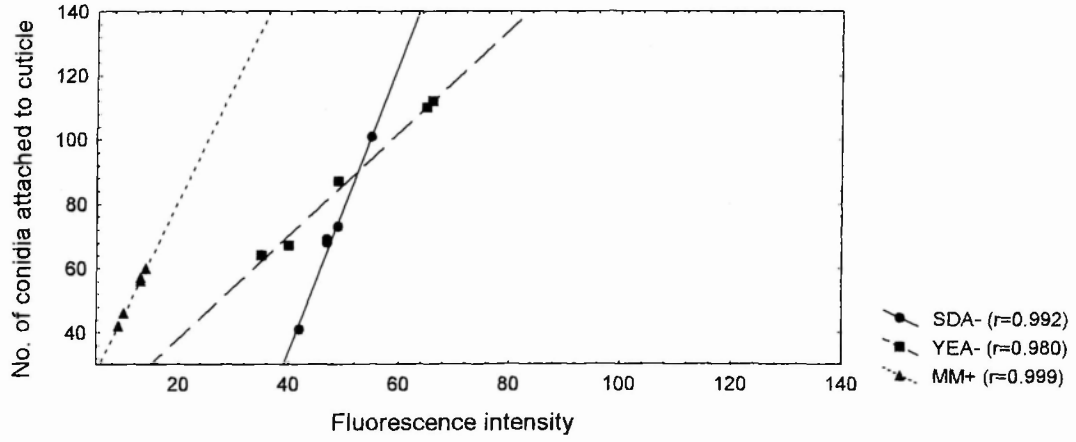
[a] SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. Numbers in parentheses are back-transformed means.

except for MM-grown conidia ($r = 0.676$, $P>0.05$). Apart from a very weak correlation ($r = 0.879$, $P<0.05$) between fluorescence and attachment of spores produced on SDA media, there were no significant relationship between these variables seen for YEA and MM-grown spores (V234). Correlation analysis was used to statistically determine the link between the above-mentioned parameters, with the findings summarised in Figure 2.3a-c. Furthermore, simple linear regression with group analysis (*via* Genstat 5) showed a significant ($F_{4,31} = 9.03$, $P<0.001$) three factor interaction, i.e. the difference in slope between isolates of the attachment-fluorescence relationship was dependent on the medium.

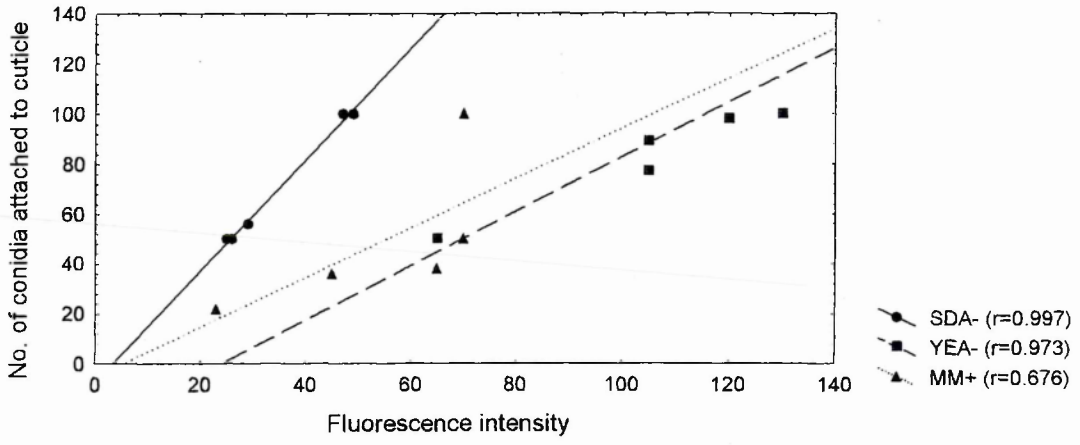
Binding of fluorochrome-conjugated lectins to the surface carbohydrates of *M. anisopliae* conidia resulted in weak, moderate or strong fluorescence (Plate 2.5e-g). The Fluorescence Index (FI) for the various treatments is summarised in Table 2.6. The data obtained from these assays were in strong agreement with the data obtained using calcofluor. Conidia from all strains exhibited very different binding affinities ($F_{2,284} = 36.27$, $P<0.001$), which were affected by substrate ($F_{7,284} = 86.67$, $P<0.001$) and depended on the lectins used ($F_{2,284} = 27.44$, $P<0.001$). High FI values were observed for Con A, WGA and RCA binding to V245 conidia from YEA- (0.60), SDAM+ (0.60) and SDA- (0.69), respectively. The highest FI values of 0.94 and 0.84 were recorded for Con A binding to V208 conidia obtained from SDAM-/SDAM+ cultures. The lowest FI values ranging from 0.02 to 0.18 were observed for Con A binding to MM-grown conidia. The results of this study have also shown a significant link between the fungal isolate and their response to culture media ($F_{14,284} = 6.49$, $P<0.001$), isolate and lectin-binding capacity ($F_{4,284} = 28.35$, $P<0.001$), and the influence of media and lectin- binding capacity

Figure 2.3a-c. Relationship between fluorescence intensity and the number of conidia attached to the cuticle of *Myzus persicae* for three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) grown on a range of culture media. •-SDA- = Sabouraud Dextrose Agar; ■-YEA- = Yeast Extract Agar and ▲-MM+ = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. Values shown in parentheses are correlation coefficient values (r).

(a)


No. of conidia attached to cuticle
140
120
100
80
60
40
20
40
60
80
100
120
140
Fluorescence intensity
SDA- (r=0.992)
YEA- (r=0.980)
MM+ (r=0.999)

(b)


No. of conidia attached to cuticle
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Fluorescence intensity
SDA- (r=0.997)
YEA- (r=0.973)
MM+ (r=0.676)

(c)

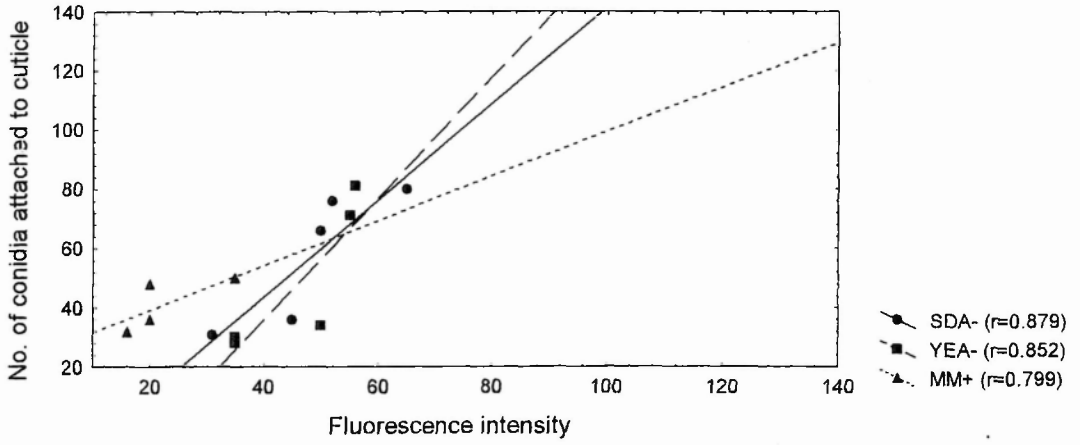

No. of conidia attached to cuticle
140
120
100
80
60
40
20
20
40
60
80
100
120
140
Fluorescence intensity
SDA- (r=0.879)
YEA- (r=0.852)
MM+ (r=0.799)

Table 2.6. Effect of culture media on Fluorescence Index[§] for the lectins Con (A), WGA and RCA bound to conidia of three isolates of *Metarhizium anisopliae.*

Medium[a]	Isolate								
	V245			V208			V234		
	Con A	WGA	RCA	Con A	WGA	RCA	Con A	WGA	RCA
SDA-	0.37 (0.01)	0.50 (0.03)	0.69 (0.10)	0.17 (0.07)	0.50 (0.07)	0.79 (0.06)	0.55(0.04)	0.47 (0.02)	0.35 (0.04)
SDA+	0.35 (0.02)	0.45 (0.18)	0.54 (0.05)	0.41 (0.03)	0.53 (0.08)	0.81 (0.04)	0.51 (0.03)	0.69 (0.03)	0.36 (0.02)
SDAM-	0.24 (0.03)	0.53 (0.03)	0.65 (0.03)	0.94 (0.03)	0.59 (0.05)	0.44 (0.04)	0.73 (0.07)	0.81 (0.04)	0.52 (0.05)
SDAM+	0.40 (0.02)	0.60 (0.09)	0.48 (0.03)	0.83 (0.02)	0.74 (0.03)	0.64 (0.07)	0.63 (0.03)	0.79 (0.02)	0.44 (0.03)
YEA-	0.60 (0.02)	0.46 (0.05)	0.34 (0.06)	0.21 (0.06)	0.55 (0.06)	0.57 (0.05)	0.57 (0.04)	0.59 (0.04)	0.43 (0.03)
YEA+	0.54 (0.07)	0.44 (0.03)	0.37 (0.06)	0.78 (0.03)	0.44 (0.06)	0.71 (0.02)	0.60 (0.03)	0.46 (0.04)	0.35 (0.02)
MM-	0.02 (0.01)	0.41 (0.06)	0.31 (0.05)	0.15 (0.04)	0.35 (0.06)	0.54 (0.09)	0.13 (0.02)	0.31 (0.07)	0.06 (0.03)
MM+	0.1 (0.03)	0.41 (0.02)	0.14 (0.03)	0.18 (0.02)	0.55 (0.06)	0.21 (0.05)	0.11 (0.03)	0.33 (0.02)	0.11 (0.02)

LSD	
Isolate	0.026 (P<0.001)
Media	0.043 (P<0.001)
Lectin	0.026 (P<0.001)
Isol*Med	0.075 (P<0.001)
Isol*Lectin	0.046 (P<0.001)
Med*Lectin	0.075 (P<0.001)
Isol*Med*Lectin	0.130 (P<0.001)

[§] Fluorescence index was calculated using the equation ((a*0) + (b*1) + (c*2) + d*3)/a+b+c+d)*(100/3) where a,b,c and d are the numbers of conidia in each of the categories: 0-no observed fluorescence, 1-weak fluorescence (photoautomat exposure of >30 min), 2-moderate fluorescence (photoautomat exposure of 1-30 min) and 3-strong fluorescence (photoautomat exposure of <1 min) as determined by a MPS 48/52 photoautomat exposure control linked to a Leitz DM-RB fluorescent microscope.
[a] SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate. Numbers in parentheses are standard errors.

of spores ($F_{14,284}$ = 14.56, P<0.001). There was also a significant three-way interaction between *Metarhizium* isolate, culture media and the lectin-binding capacity of the hydrated *Metarhizium* spores ($F_{28,284}$ = 9.38, P<0.001).

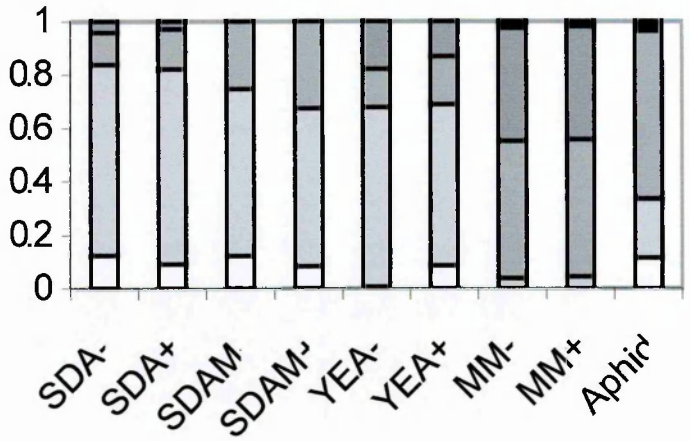
Binding of the respective lectins to the surface of fungal spores varied considerably. For example, RCA bound to localised "hot spots" on the spore surface while Con A and WGA binding was uniform. Addition of sugars specific to each lectin inhibited labelling of RCA, but only gave partial inhibition of Con A (40-60%), and WGA (>80%).

2.3.5 Polyols and trehalose content

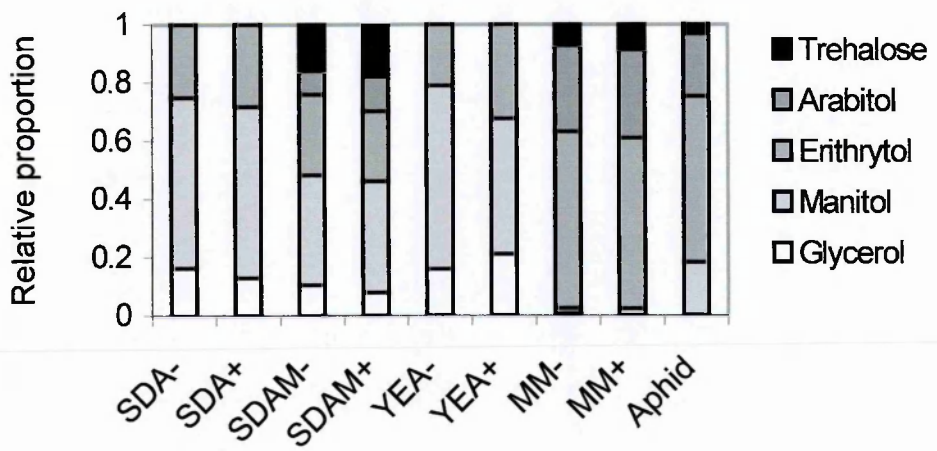
The ratios of accumulated high and low molecular weight (mol. wt) polyols and trehalose in conidia produced on different nutritional substrates are shown in Figure 2.4a-c. The quantities of these compounds varied with media (P < 0.001) and with isolate used (P < 0.001). Conidia of all three isolates produced on nutrient rich media (SDA, SDAM, and YEA) predominantly contained mannitol (mol. wt 182.2), erythritol (mol. wt 122.1) and glycerol (mol. wt 92.1), whereas conidia from nutritionally depleted media (MM) consisted mostly of erythritol, arabitol (mol. wt 152.1) and small amounts of trehalose and mannitol. Although erythritol was the major reserve of conidia harvested from aphid cadavers, other polyols and trehalose were also detected (Fig. 2.4a-c). The ability of each isolate to accumulate more specific polyols was also influenced by a two-way interaction between isolate and media (P < 0.001). For example, addition of KCl to SDA medium appeared to reduce amount of glycerol from 6.4 $\mu g\ g^{-1}$ to 5.6 $\mu g\ g^{-1}$ and from 6.9 $\mu g\ g^{-1}$ to 4.5 $\mu g\ g^{-1}$ and to significantly decrease the accumulation of mannitol from 37.3 $mg\ g^{-1}$

Figure 2.4a-c. Comparison of the ratios of polyols/trehalose present in conidia of *Metarhizium anisopliae* isolates V245 (a), V208 (b) and V234 (c) grown on a range of culture media and aphids.

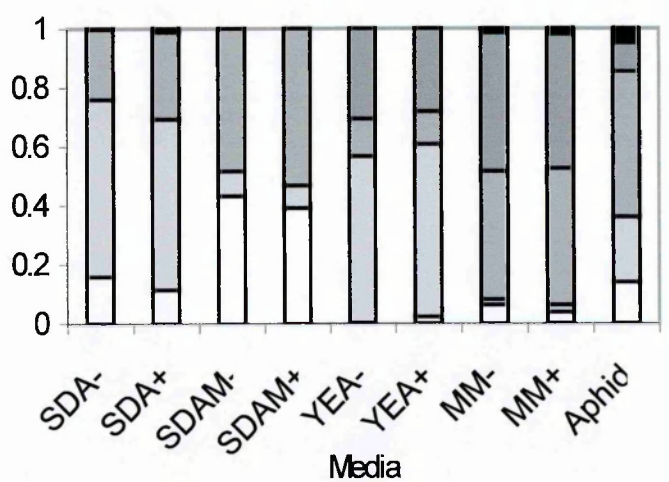
a


1
0.8
0.6
0.4
0.2
0
SDA-
SDA+
SDAM
SDAM-
YEA-
YEA+
MM-
MM+
Aphid

b


Relative proportion
1
0.8
0.6
0.4
0.2
0
SDA-
SDA+
SDAM-
SDAM+
YEA-
YEA+
MM-
MM+
Aphid
Trehalose
Arabitol
Erithrytol
Manitol
Glycerol

c


1
0.8
0.6
0.4
0.2
0
SDA-
SDA+
SDAM-
SDAM+
YEA-
YEA+
MM-
MM+
Aphid
Media

to 29.1 μg g^{-1} and from 24.7 μg g^{-1} to 16.1 μg g^{-1} in conidia of V245 and V208 isolates, respectively, as opposed to isolate V234 (Fig. 2.4a-c) where KCl induced significantly higher concentrations of glycerol and erithrytol. This medium (SDAM), however, stimulated the accumulation of greater quantities of erythritol in conidia of all three isolates tested or higher amounts of arabitol and trehalose in conidia of V208.

2.3.6 Carbon, Sulphur and Nitrogen content

Medium composition had a profound effect on the accumulation of endogenous carbon ($F_{8,52} = 12.69$, $P < 0.001$) and nitrogen ($F_{8,52} = 20.69$, $P < 0.001$) regardless of the isolate used ($F_{2,52} = 0.03$, $P > 0.05$) and slightly influenced sulphur accretion ($F_{8,52} = 2.27$, $P < 0.05$) depending on the individual organism ($F_{2,52} = 13.42$, $P < 0.001$). Statistical analysis of the data, however, revealed no significant two-way interaction between isolate and media used on total carbon, nitrogen and sulphur content. The accumulation of carbon and nitrogen in conidia of all three isolates was severely inhibited by the addition of KCl to SDA medium (Table 2.7). Highest concentrations of carbon and nitrogen (507.8 μg g^{-1} (V208) and 135.0 μg g^{-1} (V234), respectively) were detected in conidia collected from aphid cadavers. Interestingly, nutrient-deprived conditions (MM- and MM+) also prompted accumulation of higher quantities of nitrogen (103.4 and 105.2 μg g^{-1} for V245, 97.3 and 93.0 μg g^{-1} for V208, and 97.1 and 104.6 μg g^{-1} for V234, respectively). However, carbon content of the same conidia remained unchanged if compared with conidia cultivated on the nutrient-rich SDA medium (Table 2.7). Conidia harvested from YEA cultures contained less carbon than conidia from SDA or MM media, although had slightly

Table 2.7. Accumulation of endogenous carbon, nitrogen and sulphur (mg g^{-1} dry weight) in conidia of three isolates of *Metarhizium anisopliae* cultured on different media as determined by SC-144DR (Carbon/Sulfur) and FP-528 (Protein/Nitrogen) Analysers.

Media[a]	Carbon[b]			Nitrogen			Sulphur		
	Isolate								
	V245	V208	V234	V245	V208	V234	V245	V208	V234
SDA-	453.0	419.3	434.8	78.1	80.9	72.1	6.3	4.0	6.0
SDA+	465.4	472.1	444.1	79.2	81.9	70.3	6.6	4.2	5.8
SDAM-	279.7	344.2	280.1	54.0	76.6	64.3	3.7	4.8	5.6
SDAM+	285.0	319.6	288.7	55.0	77.4	62.7	2.8	4.4	5.7
YEA-	393.7	389.7	390.8	89.9	80.9	90.1	4.2	3.4	4.7
YEA+	387.3	381.4	374.9	87.9	78.4	96.1	4.3	4.1	6.6
MM-	430.6	422.5	416.7	103.4	97.3	97.1	4.5	4.7	6.6
MM+	424.1	437.9	421.0	105.2	93.0	104.6	4.9	5.5	6.6
Insect	501.9	507.8	486.5	134.1	128.8	135.0	4.9	4.3	5.0
LSD									
Isolate	31.1(P>0.05)			7.6 (P>0.05)			0.6 (P<0.001)		
Media	53.9 (P<0.001)			13.2 (P<0.001)			1.0 (P<0.05)		
Isol*med	93.3 (P>0.05)			22.9 (P>0.05)			1.8 (P>0.05)		

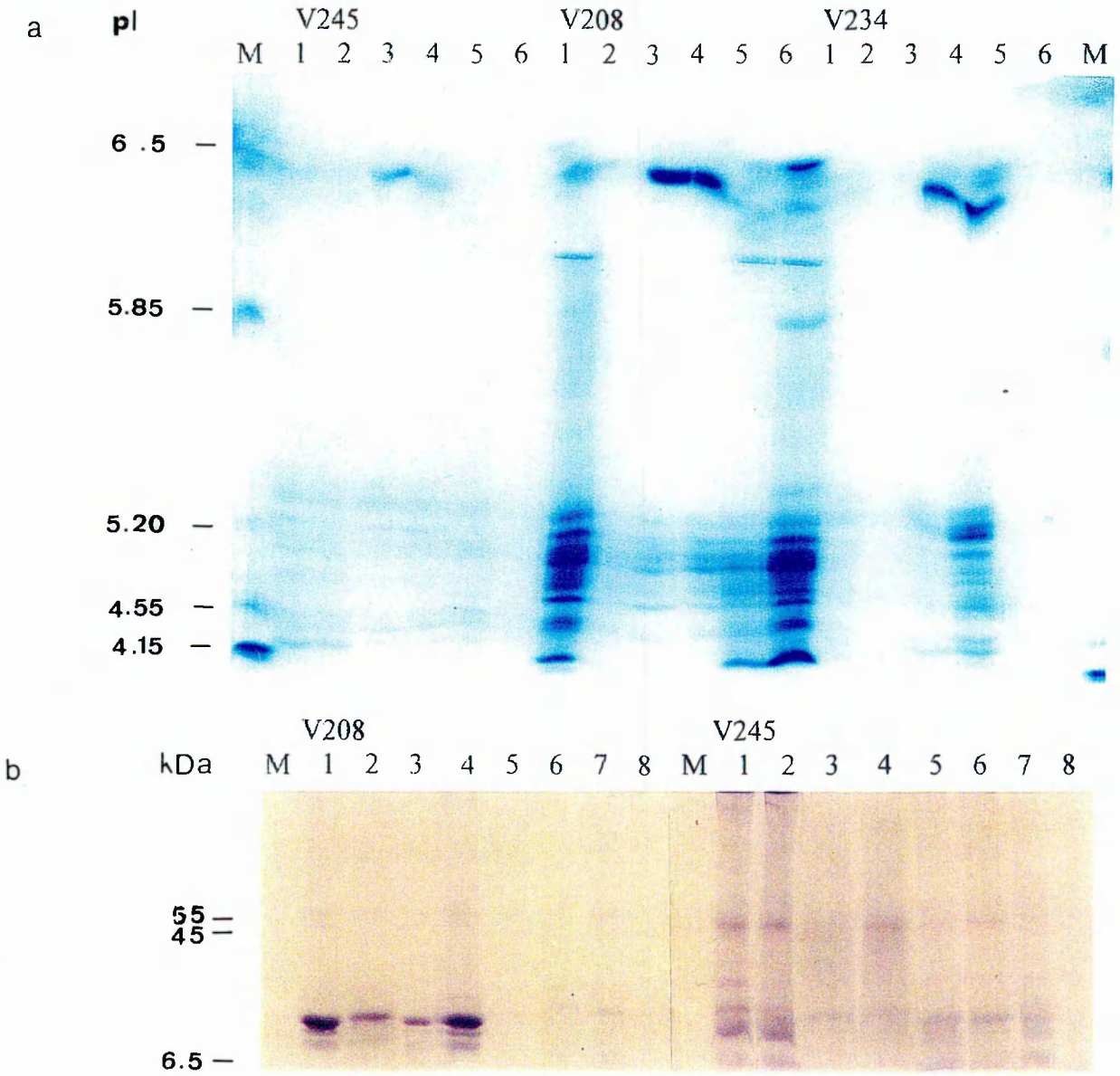
[a] SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.
[b] Each value represents the combined means of three experiments.

more nitrogen than conidia from SDA. Reduced concentrations of sulphur (2.8 μg g^{-1}) was observed in conidia of V245 produced on SDAM+ and only 3.4 μg g^{-1} of sulphur was detected in conidia of V208 grown on YEA-.

2.3.7 Electrophoretic analysis

Protein banding patterns of conidia from all three isolates examined were consistent and highly reproducible. Protein analysis using IEF (Plate 2.6a) and SDS-PAGE (Plate 2.6b) indicated not only distinct banding differences between isolates but also reflected quantitative (Table 2.8) and qualitative (Plate 2.6a-b) differences in protein profiles of conidia cultivated on different culture media. For example, conidia of all three isolates grown on SDAM- or SDAM+ media showed larger and more distinct protein bands at pI 6.53 than conidia produced on SDA. Isolate V208 also had distinctive banding patterns with two strong bands at pI 5.85 and pI 5.41 if grown on YEA+, however, these two bands were absent if aphid homogenate was excluded from the medium (Plate 2.6a). Very strong protein bands (pI 4.15) were observed in conidial extracts from SDA-, YEA- and YEA+ media but not found in extracts from other media tested. An additional, moderately strong protein (pI 4.12) band was identified only in conidia of V208 isolate cultured on SDA-. Analysis of V234 protein banding proved to be difficult because of very weak protein banding profile for almost all media tested except for SDA- and SDAM+.

Plate 2.6a-b. a. Analytical IEF (pH 4-6.5) of water-extracted proteins from conidia of three isolates of *Metarhizium anisopliae* V245, V208 and V234 harvested from SDA- (lane 1), SDA+ (lane 2), SDAM- (lane 3), S
DAM+ (lane 4),YEA- (lane 5), YEA+ (lane 6), M (pI maker proteins); **b**. SDS-PAGE of water-extracted proteins from conidia of two isolates of *Metarhizium anisopliae* V245 and V208 harvested from SDA- (lane 1), SDA+ (lane 2), SDAM- (lane 3), SDAM+ (lane 4),YEA- (lane 5), YEA+ (lane 6), MM- (lane 7), MM+ (lane 8) , M (kDa maker proteins). Protein patterned gels were stained with 0.5% Coomassie blue.


a
pI
V245
V208
V234
M 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 M
6.5
5.85
5.20
4.55
4.15
b
kDa
V208
V245
M 1 2 3 4 5 6 7 8 M 1 2 3 4 5 6 7 8
55
45
6.5

Only acidic proteins of conidia of V208 cultured on SDA or SDAM media stained bright pink, when the proteins of conidial extracts were electrophoresed on an IEF gel followed by PAS-staining, suggesting some glycosylation.

Analysis of SDS-PAGE gels also showed major protein profile differences depending on individual isolate and on the culture media used (Plate 2.6b). These differences were indicated by the presence or absence of proteins of particular size. For example, five strong bands within kDa range of 55-14.2 from SDA- and SDA+ were detected in conidial extracts of V245 isolates, yet, only two major proteins (49 kDa and 27 kDa) were recognised when the same isolate was cultured on SDA amended with KCl with or without aphid homogenate. Conidial extract of isolate V245 grown on MM exhibited over eight consistent bands within low molecular weight proteins (Plate 2.6b). In contrast, conidia of V208 produced on SDAM+ revealed more than six distinctive proteins of different size (Plate 2.6b) compared with conidia cultivated on other media tested.

Table 2.8. Mean total protein content of crude extracts obtained from conidia of three isolates of *Metarhizium anisopliae* cultured on different culture media.

Isolate	Protein (mg g^{-1} ml^{-1})[a]								
	Media[b]								
	SDA-	SDA+	SDAM-	SDAM+	YEA-	YEA+	MM-	MM+	Insect
V245	21.3	25.0	15.7	16.3	24.5	28.6	37.6	50.7	66.4
V208	27.8	21.3	26.2	26.8	21.8	33.3	53.9	45.9	57.7
V234	33.8	32.0	28.4	26.7	28.2	35.4	63.2	75.8	82.1
LSD	P<0.001								
Isolate	3.8								
Media	6.1								
Isol*med	14.7								

[b]Each value represents the combined means of four replicate cultures.
[b]SDA = Sabouraud Dextrose Agar, SDAM = SDA amended with 55 g l^{-1} KCl, YEA = Yeast Extract Agar, MM = Minimal Medium. (+) = amended and (-) = unamended with 200 mg l^{-1} of aphid homogenate.

2.4 DISCUSSION

One of the most important considerations to be taken into account when developing a commercial biocontrol agent is the determination of the culture medium that will provide optimum spore production, pathogenicity and virulence of the organism. Culture media has been shown to influence the germination, mycelial growth, morphological characters, spore yield and virulence of fungi employed as mycoinsecticides (Fargues & Robert, 1983; Lane *et al.*, 1991a) and mycoherbicides (Schisler *et al.*, 1991; Silman *et al.*, 1993; Yu *et al.*, 1998). For example, Schisler *et al.* (1991) demonstrated an improved germination and efficacy of conidia of *Colletotrichum truncatum* against *Sesbania exaltata* by producing inoculum on a medium with a low carbon/nitrogen ratio. Liquid media with a complete complement of amino acids provided an optimal nutritional environment for increased rates of conidial germination, rapid conidiation and high

conidia yield of *C. truncatum* (Jackson & Slinger, 1993). Such observations are in agreement with those obtained from this study where culture media was shown to significantly influence the germination, growth, sporulation and virulence of *M. anisopliae.*

The addition of KCl to SDA medium was shown to significantly reduce the germination of *M. anisopliae* conidia *in vitro* when compared to germination of conidia on SDA medium without KCl. Moreover, it was also observed during *in vivo* studies that conidia obtained from cultures of *M. anisopliae* grown on SDA amended with KCl had low germination and appressorial development on the cuticles of *M. persicae* and *M. aeneus.* Potassium chloride has been shown to reduce spore germination in other fungal species. For example, Cook *et al.* (1993) observed that germination of conidia of the fungal pathogen *Erysiphe graminis* was significantly lower in the presence of 0.75M KCl compared to that observed in distilled water. However, as these authors appreciated, it is difficult to determine if such a reduction in spore germination was due to an osmotic potential effect or due to possible fungicidal properties of KCl.

Apart from the inhibitory effect on germination of KCl, *in vitro* studies undertaken here also clearly demonstrated that nutrient-rich media such as SDA and YEA encouraged greater germination of *M. anisopliae* conidia than nutrient-poor media such as MM. However, such observations are in contrast to those recorded during *in vivo* studies, where conidia of *M. anisopliae* derived from cultures grown on nutrient-poor media had higher germination potential when placed on the cuticles of *M. persicae* or *M. aeneus* than conidia derived from nutrient-rich media. One possible explanation for such a discrepancy in these observations is the potential germination stimuli (Ibrahim *et al.*, 1999) or nutritional benefit offered by insect cuticles that may be lacking during *in vitro*

studies. Indeed the importance of insect material on conidial germination is exemplified by the increased percentage of germinated conidia on culture media when aphid homogenate was present.

Although strong relationships were recorded between the incidence of germinated conidia and the incidence of appressorial development on the cuticles of aphids ($r^2 = 0.5$, $P<0.01$) and pollen beetles ($r^2 = 0.71$, $P<0.01$), there was no relationship between either germination or appressorial development and LT_{50} values. This would, therefore, suggest that the virulence, as determined by LT_{50}, of *M. anispoliae* is dependent on the speed at which fungal propagules infect the host tissue rather than on the number of propagules germinating and developing appressoria. Altre *et al.* (1999) have reported that isolates of *Paecilomyces fumosoroseus* which germinated most rapidly on the cuticle of diamondback moth proved to be most virulent. Also, it has been shown that isolates of *M. anisopliae, Verticillium lecanii* and *P. fumosoroseus*, which germinated rapidly *in vitro*, proved to be more virulent towards their respective insect hosts than isolates which germinated slowly (Al-Aidroos & Roberts, 1978; Al-Aidroos & Seifert, 1980; Jackson *et al.*, 1985; Chandler *et al.*, 1993; Altre *et al.*, 1999).

The speed of germination on the other hand, could also be affected by conidial quality since the endogenous reserves of such inoculum are initially the sole source of nutrients available to conidia during the process of germination on the host surfaces. There were significant compositional differences reported in conidia of *C. truncatum* (Jackson & Schisler, 1992), *B. bassiana, M. anisopliae* and *P. fumosoroseus* (Hallsworth & Magan, 1994a, 1994b) and in blastospores of *B. bassiana* (Lane *et al.*, 1991b) produced in different nutritional environments. Moreover, conidia which contained ample endogenous nutritional reserves such as high protein and lipid content (Schisler *et al.*, 1991; Jackson

& Schisler, 1992) germinated more rapidly on water agar, whereas conidia of entomopathogenic fungi which contained more polyols, resulted in greater germination at lower water activity and caused more rapid infection and death of the larvae of *Galleria mellonella* (Hallsworth & Magan, 1994c). Also, blastospores which accumulated significantly higher levels of lipid and carbohydrates, including glycogen, have shown higher germinability and faster infection of green leafhopper than conidia with lower endogenous reserves (Lane *et al.,* 1991a 1991b). Similarly, conidia of three isolates examined in this study accumulated significantly higher levels of polyols and total proteins when grown on insect cuticles or nitrogen-limited medium (MM) than conidia cultured on YEA or SDAM media and contained higher amounts of total endogenous nitrogen (Table 2.7). Numerous studies have shown that *de novo* protein synthesis is required for initial events of germination and that amino acid pools must be available for this process, while subsequent events, such as germ tube elongation, often use exogenous sources of carbon and nitrogen (Lovett, 1976; Suzuki *et al.,* 1981; St. Leger *et al.,* 1989; Jackson & Schisler, 1992). Indeed, when total nitrogen contents of conidia of V245, V208 and V234 isolates cultured on different nutritional substrates were plotted against germinability of these isolates on aphid cuticles, the relationships were significantly linear ($r^2 = 0.91$, $P<0.001$ $r^2 = 0.68$ and $r^2 = 0.72$, $P<0.01$, respectively) (Table 2.9).

Total carbon content also appeared to influence the speed at which conidia were able to germinate on insect cuticle where more endogenous carbon induced higher germination ($r^2 = 0.86$ for V245, $r^2 = 0.93$ for V208 and $r^2 = 0.89$ for V234, $P<0.01$). Although there was no direct positive relationship found between individual polyol content and conidial germination in general (Table 2.9), statistical analysis of the data suggested that increasing glycerol content within conidial composition significantly reduced ($r^2 = 0.82$, $P<0.01$) the germination of V234. Similarly, conidia of V245 and V208 isolates, which

contained higher quantities of mannitol, germinated considerably slower ($r^2 = 0.54$ and $r^2 = 0.50$, $P<0.01$) than conidia with lower concentration of this particular polyol.

The results of this study have also indicated that those culture media which encourage the accumulation of high levels of mannitol, negatively effected conidial virulence since LT_{50} values were shown to increase as mannitol content rose ($r^2 = 0.82$, $r^2 = 0.51$ and $r^2 = 0.70$, $P<0.01$ for V245, V208 and V234, respectively). The decline in LT_{50}, however, was directly influenced by a consistent, although not significant in some cases (Table 2.9), increase in erythritol and arabitol in conidia grown on different culture medium. This would suggest that these polyols might be involved in fungal pathogenesis. Modified inocula of *B. bassiana* containing high quantities of erythritol have also resulted in rapid infection and kill of wax moths (Hallsworth & Magan, 1994c). Conidia of *M. anisopliae* collected directly from dead sporulating cadavers of mustard beetle (Magan, 2001) and conidia harvested from the cadavers of infected aphids, and highly virulent conidia from nitrogen-deprived media (MM- and MM+) in this study, contained a significantly higher amount of erythritol than conidia grown on rich culture medium such as SDA. This might

Table 2.9. Relationships between endogenous reserves accumulated in conidia of three isolates of *Metarhizium anisopliae* cultured on different media and germination *in vitro*, and between endogenous reserves and virulence of such conidia.

Isolate/Endogenous reserves (mg g^{-1} dry weight)	Germination (*in vitro*) (%)	LT_{50} (*in vivo*) (days)
V245		
C	$R^2 = 0.86$; $P<0.01$	$R^2 = 0.00$
N	$R^2 = 0.91$; $P<0.001$	$R^2 = 0.22$
C:N	$R^2 = 0.18$	$R^2 = 0.50$; $P<0.01$
Glycerol	$R^2 = 0.38$	$R^2 = 0.00$
Mannitol	$R^2 = 0.13$	$R^2 = 0.82$; $P<0.01$
Erythritol	$R^2 = 0.54$; $P<0.01$	$R^2 = 0.69$; $P<0.01$
Arabitol	$R^2 = 0.47$	$R^2 = 0.71$; $P<0.01$
Total polyols and trehalose	$R^2 = 0.48$	$R^2 = 0.68$; $P<0.01$
V208		
C	$R^2 = 0.93$; $P<0.001$	$R^2 = 0.10$
N	$R^2 = 0.68$; $P<0.01$	$R^2 = 0.22$
C:N	$R^2 = 0.00$	$R^2 = 0.60$; $P<0.01$
Glycerol	$R^2 = 0.20$	$R^2 = 0.65$; $P<0.01$
Mannitol	$R^2 = 0.10$	$R^2 = 0.51$; $P<0.01$
Erythritol	$R^2 = 0.50$; $P<0.01$	$R^2 = 0.40$
Arabitol	$R^2 = 0.44$	$R^2 = 0.50$; $P<0.01$
Total polyols and trehalose	$R^2 = 0.52$; $P<0.01$	$R^2 = 0.41$
V234		
C	$R^2 = 0.84$; $P<0.01$	$R^2 = 0.09$
N	$R^2 = 0.72$; $P<0.01$	$R^2 = 0.16$
C:N	$R^2 = 0.00$	$R^2 = 0.51$; $P<0.01$
Glycerol	$R^2 = 0.82$; $P<0.01$	$R^2 = 0.03$
Mannitol	$R^2 = 0.05$	$R^2 = 0.70$; $P<0.01$
Erythritol	$R^2 = 0.06$	$R^2 = 0.48$
Arabitol	$R^2 = 0.46$	$R^2 = 0.39$
Total polyols and trehalose	$R^2 = 0.27$	$R^2 = 0.21$

explain why inocula directly isolated from the body of the dead insects were often more virulent against targeted pests than those obtained from the cultures produced on nutrient-rich artificial media.

Studies with other fungi have suggested that carbon and nitrogen concentrations, and C:N ratios of nutritional conditions are responsible for the pathogenicity of resultant inocula (Hallsworth & Magan, 1994a; Jackson & Bothast, 1990; Schisler *et al.*, 1991). However, no link has ever been established between the C:N ratio of such inoculum and virulence. Results obtained from these studies, demonstrate for the first time, that the onset of initial infection processes may be directly related to C:N ratio of infective propagules since the level of virulence, as measured by LT_{50} values, significantly increased as the C:N ratios in conidia of each isolate decreased ($r^2 = 0.50$ (V245), $r^2 = 0.60$ (V208) and $r^2 = 0.51$ (V234), $P<0.01$).

The speed at which a fungal pathogen is able to infect and penetrate its host will be to some degree influenced by the ability of the infecting propagule to securely adhere to the host. In studies undertaken here, where conidia of *M. anisopliae* were used to inoculate pollen beetles, the virulence of isolate V245, determined by LT_{50} values, was strongly related to the number of conidia which had successfully adhered to the surface of the cuticle ($r^2 = 0.54$, $P<0.01$). Furthermore, the culture medium from which conidia of *M. anisopliae* were derived was shown to significantly influence the adherence of conidia to insect cuticle. The role of hydrophobic forces, mucilage and lectins in the recognition and adhesion of fungal spores to host substrata is well documented (Fargues, 1984; Boucias & Latgé, 1986). It would appear, therefore, that the culture media used in this study influenced the surface properties of conidia produced.

Indeed, in the majority of cases, a strong linear relationship was established between fluorescence intensity of calcofluor treated conidia and the number of spores that adhered to the cuticle of aphids (Fig. 2.8a-c) where increasing spore fluorescence increased spore adhesion. Since calcofluor binds to 1-4-β-glucans (Butt *et al.*, 1989), then it would appear that these polysaccharides play an important role in either host recognition or spore adhesion for *M. anisopliae*. Surface glucans produced by *Rhizobium* spp. have been shown to play an important role in the recognition of leguminous host plants by the bacteria (van der Drift *et al.*, 1998; Price, 1999; Spaink, 2000). Glucans have also been shown to be important in determining the adhesion of oral *Streptococcus* species to teeth and gums (Doyle & Taylor, 1994).

Although no relationship could be established between the binding of lectins Con A, WGA and RCA to conidia and spore adhesion, significant differences in lectin binding was observed for each of the culture media tested suggesting that culture media affects the surface carbohydrates of fungal spores. However, since there was no direct relationship between lectin binding and spore adhesion, it is difficult to determine from this study the relative importance of carbohydrates such as α-D-mannose, α-D-glucose, β-D-galactose and N-acetyl-D-glucosamine in spore adhesion.

Surface carbohydrates are known to be positively correlated to surface hydrophocity (Jeffs *et al.*, 1999). It could be suggested that observations on spore adhesion recorded in this study were a reflection of culture media influencing surface carbohydrates of *M. anisopliae* conidia, which in turn influenced binding of spores to insect cuticles by hydrophobic forces. However, it must be remembered that spore adhesion may not be entirely dependent on the attributes of the pathogen but is also influenced by the nature of

the host cuticle. For example, Butt *et al.* (1995) showed that fungistatic compounds, present in the host epicuticular waxes, also influenced spore adhesion.

Metarhizium anisopliae has been shown to be an effective biocontrol agent against a number of insect pests both under laboratory and field conditions (Butt *et al.*, 1992; 1994; 1995; 1998). Reduction in pathogen virulence, also known as attenuation, resulting from continued inoculum production on artificial culture media, is a phenomenon well documented (Fargues & Roberts, 1983; Morrow *et al.*, 1989; Butt & Goettel, 2000). Results from this study have also identified that culture media influences the virulence of *M. anisopliae* isolates by influencing the fungal physiological qualities such as presence or absence of erythritol and arabitol and the balance of carbon and nitrogen (C:N ratios) in conidia. The level of virulence was also affected by the ability of conidia to adhere to the cuticle of aphids and beetles. Furthermore, it has been identified that adhesion of *M. anisopliae* conidia to insect cuticles is affected by the amount of β-glucans on spore surfaces. Since the intensity of calcofluor fluorescence of stained conidia correlated well with conidial adhesion to host cuticles in these studies, the author propose that such a simple vital stain assay could prove useful in determining the potential virulence of produced inoculum, whether it be for further laboratory studies or commercial production.

CHAPTER 3

The effects of successive *in vitro* sub-culturing on morphological and physiological changes in the entomopathogenic fungus, *Metarhizium anisopliae*

3.1 INTRODUCTION

Attenuation can result from a single sub-culture or repeated sub-culturing on artificial media (Kawakami, 1960; Nagaich, 1973). This phenomenon is often associated with morphological changes (Hall, 1980, Lord & Roberts, 1986). For example, Kawakami (1960) examined the effects of successive sub-culturing of several pathogens of silkworm larvae, including *Spicaria pracina, Isaria fumosorosea, Oospora destructor, Aspergillus flavus, Aspergillus oryzae* and *Beauveria bassiana* on culture media including pupae-decoction agar (Pd), plain agar (A) and sterilised silkworm pupae (P). Cultures were started from a single spore and 19 generations were repeated taking about 30-180 days for each generation. Cultural changes such as a decrease of growth rate, amount of aerial mycelium, formation of coremia and amount of sporulation and change of colony colour were marked. These tendencies were more remarkable with each successive sub-culture for almost all species tested. The exceptions, however, were *B. bassiana* and *A. flavus*, where the former showed no change at all while the latter's spore production increased with repeated sub-culturing on Pd medium. These variations disappeared following passaging on silkworm body. Similar morphological changes associated with attenuation have also been observed for *E. neoaphidis* (Wilding *et al.*, 1992) and *N. rileyi* (Lord & Roberts, 1986; Morrow *et al.*, 1989). Hall (1980) has reported physiological and morphological changes in single and multi-spored isolates of a *Verticillium lecanii* strain after repeated sub-culturing on different agars. The changes, however, did not obtain full reversion to the parental morphology after passaging once through *Macrosiphoniella sanborni*.

The aims of this study were to (i) determine the morphological changes associated with repeated *in vitro* passaging of three isolates *of Metarhizium anisopliae* on two different nutritional substrates and (ii) determine physiological factors, which may account for the attenuation observed.

3.2 MATERIALS AND METHODS

3.2.1 Origin and maintenance of *Metarhizium anisopliae* isolates

Three isolates of *Metarhizium anisopliae* conidia (see Chapter 2, section 2.2.2) were re-hydrated in 0.03% Tween 80 (BDH) for 30 min and placed on SDA (Lab M, UK) before being incubated in dark at 23 ± 1°C for 14 days. Following incubation, conidia of each isolate were harvested from respective sporulating colonies and suspended in 0.03% Tween 80. Concentrations of conidial suspensions for each of the three isolates were then determined using an "Improved Naubeaur" haemocytometer and adjusted to a final concentration of 10^7 conidia ml^{-1}. Each isolate was passaged through adult peach-potato aphids (*Myzus persicae*) by immersing aphids in 5 ml of conidial suspension for 10 seconds. Excess conidial suspension was removed by placing inoculated aphids onto filter paper in a Buchner funnel. The aphids were then placed into 90 mm Petri dishes containing 20 ml of water agar (WA, Lab M, UK) on which a 50 mm diameter Chinese cabbage leaf disc was placed. Aphids were then incubated at 23 ± 1°C under a 16 h photoperiod until they were observed to have died. The collection of conidia from sporulating cadavers allowed virulent inoculum of each isolate to be obtained. It was this unattenuated inoculum that provided the primary inoculum for subsequent studies of attenuation in each of the three isolates of *M. anisopliae*.

For each isolate of *M. anisopliae* tested, conidia collected from sporulating cadavers (primary inoculum) were used to inoculate Petri dishes containing either SDA or Minimal Media ((MM: 1.5% (w/v) Agar No.1 (Lab M, UK), 0.03% K_2HPO_4, 0.03% $MgSO_4.7H_2O$, 0.015% NaCl, 0.03% $CaCl_2.6H_2O$ (saturated), 0.0008% $MnSo_4.4H_2O$, 0.0002% $CuSO_4$ $5H_2O$ and 0.002% $FeSO_4.7H_2O$). Following incubation in dark at 23°C for 14 days, the resultant sporulating colonies (sub-culture 1) provided inoculum for the inoculation of further plates of SDA and MM. The sequence of successive sub-culturing was repeated until each isolate had been sub-cultured 11 times on both SDA and MM.

3.2.2 Assessments of *in vitro* germination, growth and sporulation

For each isolate grown on SDA and MM, conidia were harvested from sub-cultures 1,3,5,7,9 and 11 and suspended in 0.03% v/v aqueous Tween 80. Ten µl of conidial suspension (10^5 conidia ml^{-1}) was then dispensed onto the centre of ten replicate Petri dishes containing 15 ml of SDA. All plates were sealed with parafilm 'M' (American National) and incubated at 23° in dark. After 24 h incubation, five replicate plates of each isolate and sub-culture were removed and the incidence of germinated conidia recorded for five hundred conidia (100 conidia per replicate). Conidia were considered to have germinated when the germ-tube was as long as the conidium was wide (Manners, 1966). The remaining five replicates were examined at 3, 6, 9 12, and 15 days, when the morphology and phenotypic characters (colour, exudates, sectors) of mycelial colonies were assessed and the diameter of colonies measured. The same replicate plates were used to estimate the number of conidia produced by each isolate and sub-culture. Three plugs were randomly taken from each of the five plates using a

No. 3 cork borer (6 mm in diameter). Each plug was then placed into 1.9 ml micro centrifuge tube containing 1 ml 0.03% Tween 80. The tubes were vigorously shaken for 30 min (Friffin Ltd, UK) and conidial counts were performed using the haemocytometer.

3.2.3 Electrophoretic studies

Electrophoretic studies were conducted on isolateV245 only, since phenotypic changes associated with *in vitro* passaging through SDA medium were greater in this isolate than in V208 or V234 isolates. To see whether morphological/phenotypic changes manifest themselves in the differential production of protein (different quantities or new proteins produced), fresh aerial conidia of isolate V245 from sub-cultures 1,3,5,7,9,11 and conidia isolated directly from aphid cadavers (see section 2.2.8), and conidia together with mycelia from white or yellow cultures of sub-culture three were electrophoresed for esterase profile. Total protein was extracted from 10 mg of conidia or conidia and mycelia by suspending them in homogenisation buffer consisting of 2 ml 20% v/v Glycerol, 200 µl 2% w/v Triton X100 and 0.01 g bromophenol blue made up to a final volume of 10 ml with distilled water. Samples were then homogenized in Eppendorf tubes for 45 s in an ice bath and subsequently centrifuged at 10, 000 rpm for 5 min at 4° C. The clarified supernatant (10 µl) was immediately loaded on the native polyacrilamide (7% resolving and 3% stacking) gel and run for 50 min at 15 mA/gel and 4° C in a standard gel apparatus (Atto, Japan). After electrophoresis, the gel was incubated in esterase substrate consisting of 4% w/v α, β–naphthyl acetate or α-naphthyl butyrate (Sigma, UK) dissolved in anhydrous acetone and diluted to 0.04% w/v in 0.1M Tris-HCl, pH 7.9 in which 0.2% w/v Fast Blue RR salt was dissolved. The gels were

incubated at room temperature until bands were sufficiently developed (Loxdale *et al.*, 1983). The reaction was stopped by adding 10% acetic acid. Relative electrophoretic mobility (Rm) for each enzyme band was calculated as the ratio of its movement to that of the marker dye.

3.2.4 Assessments of *in vivo* adhesion, germination, differentiation of appressoria and sporulation

Conidial suspensions used for *in vitro* studies (section 3.2.2) were also used to investigate the ability of these conidia to adhere, germinate, produce appressoria and sporulate on aphid cadavers. For each of the prepared conidial suspensions, 10 adult aphids were inoculated by immersing them for 10 seconds in 5 ml of suspension. Excess conidial suspension was removed by placing inoculated aphids onto filter paper in a Buchner funnel. Ten aphids were then transferred to a leaf disc (50 mm diameter) of Chinese cabbage placed in a 9 cm diameter Petri dish lined with moist tissue paper. After the 24-hour-incubation period identical to that described previously (section 2.2.4), 30 aphids (three replicate dishes) were frozen at -20° C before being prepared for fluorescence and SEM examination. Adhesion, germination and germling development on aphid cuticle was recorded using fluorescence and SEM methods described previously (section 2.2.4). Measurements of spore dimensions, length and width of the germlings and appressoria were done using image analyser (Coolview II, Ophotonic Science Ltd., UK). The remaining three dishes containing ten aphids were left to incubate under the same conditions as described above and examined daily for dead insects over a period of seven days. Dead individuals were transferred to Petri dishes lined with moist tissue paper and subjected to the same incubation procedures described

above in order to encourage fungal growth and sporulation. Following incubation, the number of sporulating cadavers was recorded for each replicate.

3.2.5 Adhesion of conidia and germlings to artificial surface

Metarhizium anisopliae was also examined for its ability to adhere to a polystyrene surface (Petri dishes, Sarstedt, Germany). For isolate V245 grown on SDA, conidia were harvested from sub-cultures 1,3,5,7,9 and 11 and washed three times in 0.03% (v/v) aqueous Tween 80. Free from mycelial debris, conidial pellets were then re-suspended in 1ml of either: distilled water (DW) or 1% (w/v) glucose (Sigma, UK) in DW (DWG), 1% (w/v) yeast extract (YE, Sigma, UK) in DW (DWYE) and 1% (w/v) glucose in DWYE (DWYEG). A 100 µl droplet of each conidial suspension, containing about 200 conidia, was placed on the artificial test surface and incubated in moisture chambers. At intervals of 24, 48 and 72 hr after inoculation, samples were removed, and conidia counted using differential interference contrast optics within a circular area (3 mm diameter) drawn on the test surface. Samples were then exposed to a standard washing procedure in which 5 ml of DW (approximately 70 drops) were delivered from a height of 10 cm onto the inoculated surface held at a 45° angle. After washing, conidia were stained with 0.001% w/v calcofluor (Sigma) and the number of conidia remaining within the designated area was determined using the fluorescence microscope.

3.3 RESULTS

3.3.1 Germination, growth and morphological phenotype of the three *M. anisopliae* isolates *in vitro*

Repeated passaging of *M. anisopliae* isolates through SDA medium significantly affected germination of resultant conidia, speed of growth and phenotype. Substantial differences in % germination of conidia were observed between isolates ($F_{2, 132}$=663.93) with conidia of isolate V245 having a slightly lower rate of conidial germination than V234 isolate (Table 3.1) but considerably higher than V208. Each isolate also exhibited different responses to repeated sub-culturing ($F_{10, 132}$=91.06). For example, a slight reduction in germination was noted for sub-cultures 3, 5 and 11 for isolate V234. Sub-culturing also less germination for 3rd and 9th generations of isolate V245 by 20% and 19%, respectively (Table 3.1). Only 20% and 43% of conidia had germinated in sub-cultures 7 and 3, respectively, for isolate V208. Statistical analysis revealed a significant effect of repeated sub-culturing on spore germination of the respective isolates ($F_{20, 132}$=15.09). However, 100% germination was observed for conidia harvested from sub-cultures of all three isolates on MM media (Table 3.1).

Table 3.1. Effect of *in vitro* sub-culturing on the germination of three isolates of *Metarhizium anisopliae* following 24 h incubation at 23°C on SDA.

Isolate	Sub-culture	Percentage germination (±SE)	
		SDA	MM
V245	1	96 (1.1)	100 (0.0)
	3	80 (2.1)	100 (0.0)
	5	93 (1.4)	100 (0.0)
	7	95 (1.1)	100 (0.0)
	9	81 (2.1)	100 (0.0)
	11	97 (0.9)	100 (0.0)
V208	1	93 (1.4)	100 (0.0)
	3	43 (2.7)	100 (0.0)
	5	67 (2.5)	100 (0.0)
	7	20 (2.1)	100 (0.0)
	9	65 (2.6)	100 (0.0)
	11	85 (1.9)	100 (0.0)
V234	1	99 (0.6)	100 (0.0)
	3	88 (1.7)	100 (0.0)
	5	87 (1.0)	100 (0.0)
	7	98 (0.8)	100 (0.0)
	9	97 (1.8)	100 (0.0)
	11	90 (0.6)	100 (0.0)

Data from a comparative study on the growth of *M. anisopliae* isolates on SDA medium showed that V208 grew faster (Fig. 3.1) than isolates V234 and V245. Although the rates of fungal growth were only slightly affected by sub-culturing, the overall colony

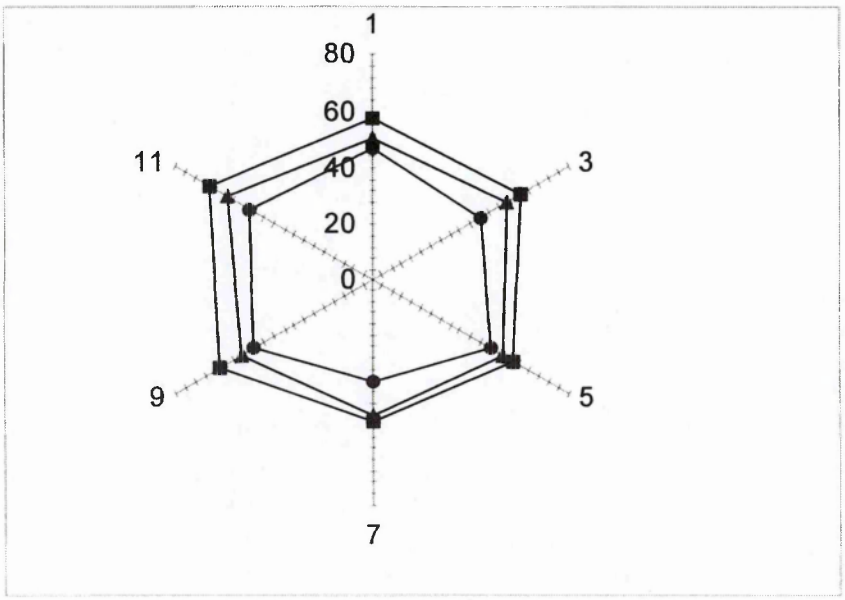


Figure 3.1. Effect of sub-culturing on colony diameter (mm) of three isolates of *Metarhizium anisopliae* ; V245 (●), V208 (■) and V234 (▲) following incubation for 15 d at 23° C on SDA (Sabouraud Dextrose Agar). 1,3,5,7,9,11 are successive sub-cultures. Each data point is the mean of five replicate cultures.

sizes after 15 d incubation at 23° C, significantly differed from each other ($F_{9, 261}$=255.93) (Fig. 3.1). Radial growth of isolate V208 was greater for sub-cultures 3, 9 and 11 resulting in final colonies of 6 cm diameter, whereas the greatest colony size of V234 and V245 was measured for sub-culture 11 with diameters of 5.9 cm and 4.9 cm, respectively. The slowest fungal colony growth was observed for sub-culture 7 (3.6 cm) of isolate V245 (Fig. 3.1). In contrast, all sub-cultures of all three isolates tested on MM

medium grew at similar rates and produced colonies of similar diameter after 15 days of incubation at 23° C.

The onset of fungal sporulation was first observed after 3 days incubation on SDA medium for sub-cultures 1, 5 and 9 of isolate V245. In contrast, for isolate V234, sporulation started in all sub-cultures following 3 d incubation. Six days post-inoculation, one sector was recorded in one of the five replicates for sub-cultures 5 and 11 of isolate V245, and two sectors in one of the five replicates for sub-culture 5 of isolate V234. The number of cultures producing sectors and the number of sectors occurring in each culture, increased with time of incubation (Table 3.2). Most of the recorded sectors appeared to be conidia-free or produced very few conidia.

Successive sub-culturing of *M. anisoplae* inocula on SDA medium also caused isolates V208 and V245 to become sterile. The first sterile culture of V208 appeared among replicates in sub-culture 3 (Plate 3.1). The number of sterile replicates increased in frequency with each consecutive sub-culture. Sporulation of the sterile mycelium was, however, initiated by scraping it with a sharp needle. Conidia induced by mechanical damage gave rise to a sterile mycelium in all subsequent sub-cultures. Colonies of V245 which developed areas of both aerial mycelia and conidia, were first observed in sub-culture 3, which in turn, gave rise to sterile colonies appearing in the following sub-cultures. In addition, attenuated sub-cultures 3 and 9 of V245 formed colonies as a mixture of white and/or yellow mycelia and spores of uncharacteristic pale green colour. Increasing the number of sterile sectors or sterile colonies amongst the replicates also influenced total conidial yield (Table 3.3). By contrast, isolate V234 consistently sporulated profusely producing a thick mat of typical dark-green conidia.

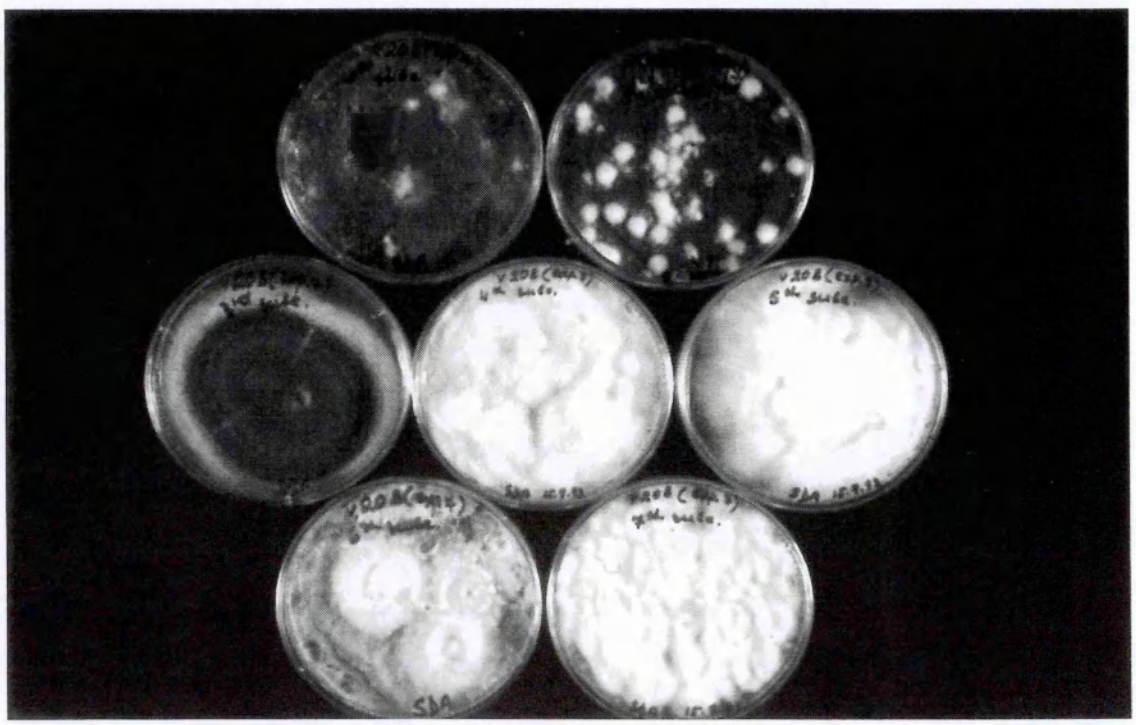


Plate 3.1. Successive sub-culturing of *Metarhizium anisopliae* on SDA caused isolate V208 to become sterile. Note that attenuated sub-cultures 4, 5, 6 and 7 produced white mycelium only.

Passaging *M. anisopliae* through MM media eleven times in succession, however, made no difference on conidial production of the three isolate observed.

Table 3.2. Effect of *in vitro* sub-culturing on the phenotype of three isolates of *Metarhizium anisopliae* following 15 d incubation at 23°C on SDA (10 replicate cultures).

Isolate	Sub-culture	No of sterile cultures	No of Sectors	Pigmentation of Mycelia[a]	Substrate Pigmentation[b]
V245	1	0	8		P (2)
	3	0	18	W (1), Y (3)	P (6)
	5	2	8	W (4), Y (1), S (3)	
	7	2	2	Y (2), S (3)	
	9	3	10	Y (5), S (5)	P (4)
	11	5	2	S (4)	
V208	1	0	0		
	3	1	3	S (4)	
	5	4	4	S (6)	P (2)
	7	6	0	S (7)	
	9	7	0	S (5)	
	11	9	0	S (7)	
V234	1	0	0		
	3	0	0		
	5	0	1		
	7	0	2		
	9	0	4		
	11	0	0		

[a] -number of replicate cultures producing patches of white (W) and yellow (Y) or sterile (S) mycelia (n=10).
[b]- number of replicate cultures releasing pink (P) pigments into nutrient substrate (n=10).

Table 3.3. Effect of *in vitro* sub-culturing on SDA on conidia production by three isolates of *Metarhizium anisopliae* 15 days after inoculation.

Sub-culture	10^7 conidia ml^{-1}[a]		
	Isolate		
	V245	V208	V234
1	9.43	7.23	8.53
3	4.50	2.43	7.50
5	9.33	2.30	8.63
7	8.33	4.63	9.60
9	5.41	4.50	8.40
11	6.60	6.60	8.13
LSD[b]	(P< 0.001)		
Isolate	0.50		
Sub-cult.	0.70		
Isol*Sub-cult.	1.22		

[a]Each value represents the combined means of three replicate cultures.
[b]Least significant difference.

3.3.2 Isozyme electophoresis

Separation of multiple forms of non-specific α, β-esterase isozymes by native gel electrophoresis was consistent and highly reproducible for all samples tested. Conidia of isolate V245 harvested from mycosed aphids had a characteristic esterase pattern consisting of isozyme bands of different mobility: five strong bands at Rm = 0.15, 0.22, 0.43, 0.53 and 0.75 and two moderately strong bands at Rm = 0.18 and 0.65 (Plate 3.2a). Esterase profile of sub-culture 1 harvested from SDA medium was very similar to that observed in the extracts collected from conidia of aphid cadavers. Esterases at Rm = 0.15, 0.22 and 0.65, however, exhibited bands of lesser intensity than those of parental bands (Plate 3.2a, Lane 1). There were virtually no bands observed in the conidial extract of sub-culture 3 grown on SDA medium (Plate 3.2a) with the exception

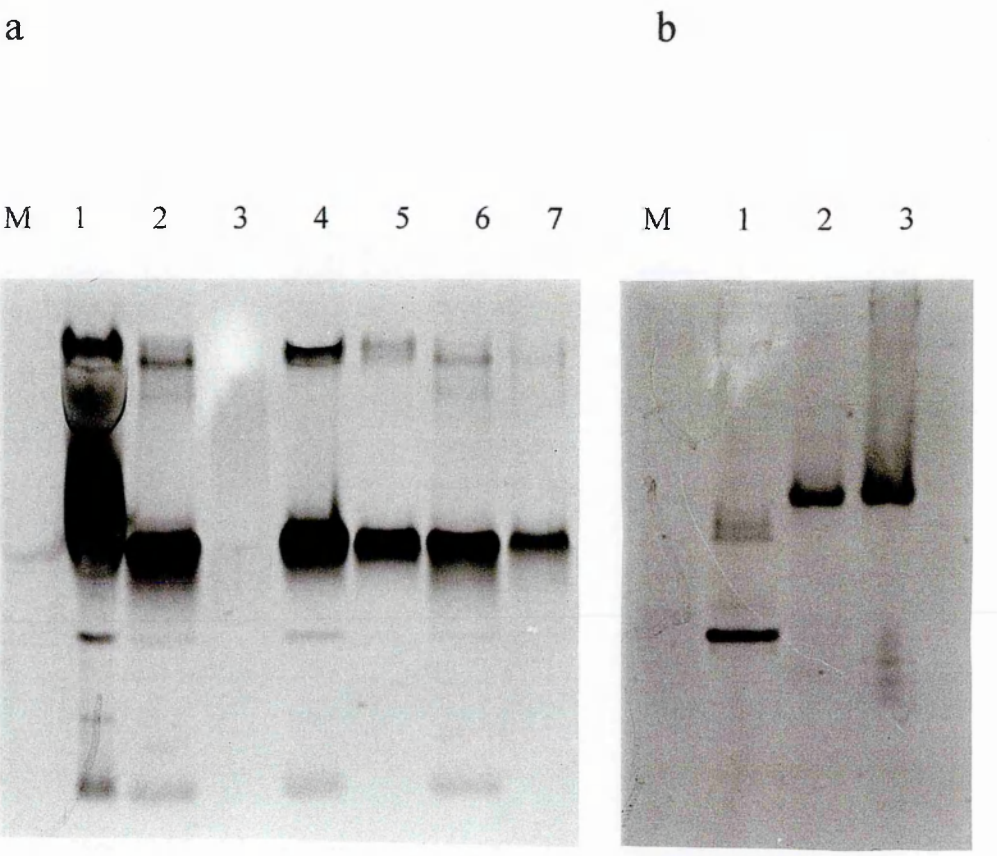


Plate 3.2 a-b. Comparative non-specific esterase banding patterns of isolate V 245 separated by slab gel electrophoresis: **a.** conidia harvested from sporulating aphid cadavers (lane 1) and from profusely sporulating sub-cultures: one (lane 2), three (lane 3), five (lane 4), seven (lane 5), nine (lane 6) and eleven (lane 7); **b.** conidia and mycelia harvested from sub-culture three: morphologically similar to sub-culture one (lane 1), producing white mycelia (lane 2) and producing yellow mycelia (lane 3). M (marker dye). Rm (relative electophoretic mobility).

of an inconsistent extra weak band at Rm = 0.43. The bands at Rm = 0.22 were absent in the populations of sub-cultures 3, 5 and 11 and relative mobility bands of 0.65 were also absent in conidia of sub-cultures 7, 9 and 11. Sub-cultures 7 and 11 also lacked esterase isozyme at Rm = 0.75. However, sub-culture 9 showed an additional weak esterase band at Rm = 0.39 (Plate 3.2a). Conidia and mycelia from sub-culture 3 were also tested for α-naphthyl butyrate activity (Plate 3.2b). Conidia collected from the cultures which appeared morphologically similar to those of non-attenuated cultures, hydrolysed the α-butyrate substrate resulting in 4 prominent bands at Rm = 0.10, 0.31, 0.32 and 0.45. In contrast, conidia harvested from white mycelia exhibited one strong and one weak band of Rm = 0.29 and Rm = 0.21, respectively, whereas conidia from yellow mycelia had a different esterase pattern consisting of a band of strong intensity at Rm = 0.29 and three bands of lesser intensity at 0.50, 0.52 and 0.53.

3.3.3 The germination behaviour of the three *M. anisopliae* isolates *in vivo*

Germination behaviour of all three isolates examined and their response to repeated passaging through SDA and MM media are presented in Tables 3.4 and 3.5, respectively. Whilst interpreting the results of this study, it was important to note the following features: fungal isolate ($F_{2,315}$=14.83, P<0.001) and source of conidial inocula ($F_{1,315}$=71. 36, P<0.001) affect adherence; the number of *M. anisopliae* conidia of each sub-culture that bind to aphid individuals was variable ($F_{5,315}$=20.97, P<0.001). This variability was directly affected by individual responses of each isolate to both substrates ($F_{2,315}$=28.36, P<0.001) and repeated sub-culturing ($F_{10,315}$=6.70, P<0.001). The attachment of conidia from each sub-culture was also influenced by the media

Table 3.4. Percentage of germinated conidia and of germlings which had produced appressoria on the cuticle of *Myzus persicae* individuals 24 h post-inoculation for three isolates of *Metarhizium anisopliae* sub-cultured on SDA and MM artificial media.

Isolate/ Sub-culture	Media					
	SDA			MM		
	Conidia (n)[a]	Germinated (%)[b]	Appressoria (%)[c]	Conidia (n)	Germinated (%)	Appressoria (%)
V245						
1	2.7	90.0 (2.8)	12.1 (1.9)	30.1	100.0 (0.0)	3.8 (1.0)
3	4.2	54.2 (8.8)	0.0 (0.0)	72.4	100.0 (0.0)	7.8 (0.6)
5	1.9	90.4 (4.7)	10.0 (2.0)	58.9	100.0 (0.0)	24.2 (3.2)
7	7.0	100.0 (0.0)	4.5 (0.4)	34.1	100.0 (0.0)	18.3 (1.8)
9	18.1	76.2 (1.2)	0.0 (0.0)	85.8	100.0 (0.0)	4.6 (0.7)
11	5.5	87.9 (3.7)	0.0 (0.0)	28.5	100.0 (0.0)	12.2 (1.4)
V208						
1	5.2	100.0 (0.0)	12.4 (1.1)	7.9	100.0 (0.0)	13.1 (1.1)
3	30.5	44.3 (2.5)	2.8 (0.8)	8.3	100.0 (0.0)	10.8 (1.3)
5	18.3	85.6 (2.5)	0.0 (0.0)	3.0	100.0 (0.0)	0.0 (0.0)
7	70.9	73.4 (3.9)	0.0 (0.0)	4.4	100.0 (0.0)	10.5 (1.9)
9	27.9	82.8 (3.1)	3.8 (0.9)	80.0	100.0 (0.0)	14.0 (0.6)
11	2.9	92.5 (4.9)	15.4 (1.7)	12.8	100.0 (0.0)	12.5 (1.3)
V234						
1	18.0	95.0 (2.2)	17.9 (1.3)	16.9	100.0 (0.0)	48.3 (4.6)
3	39.8	59.0 (3.3)	8.0 (1.0)	59.8	100.0 (0.0)	14.0 (1.1)
5	38.1	76.2 (3.0)	10.5 (0.9)	30.1	100.0 (0.0)	33. 3 (2.7)
7	23.1	89.6 (2.7)	16.0 (1.2)	109.7	100.0 (0.0)	8.3 (0.7)
9	4.5	95.7 (4.0)	7.1 (0.7)	127.9	100.0 (0.0)	7.3 (0.9)
11	16.3	70.1 (4.9)	20.0 (1.5)	25.8	98.1 (1.1)	20.3 (1.3)
LSD	P<0.001					
Isol.	7.32					
Med.	5.98					
Sub-cult.	10.35					
Isol*Med.	10.35					
Isol*Subcult.	17.93					
Med*Subcult.	14.64					
Isol*Med*Subc	25.36					

[a] Total number of conidia adhered at the dorsal site of conidial application.
[b] Percentage of observed conidia which had germinated. Numbers in parentheses are standard errors (SE).
[c] Percentage of germinated conidia which had produced appressoria. Numbers in parentheses are SE.

Table 3.5. Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA and MM artificial media on conidia, germ-tube and appressorium dimensions following incubation at 23°C for 24 h.

Isolate/ Sub-culture	Media SDA Conidium		Germtube		Appressorium		MM Conidium		Germtube		Appressorium	
	L[a]	W[b]	L	W	L	W	L	W	L	W	L	W
V245												
1	5.3	3.2	12.0	1.3	5.7	3.1	5.2	2.7	27.2	1.4	5.6	3.4
3	5.5	2.9	19.1	1.7	-	-	5.6	3.0	23.7	1.5	4.9	3.2
5	6.0	3.1	12.1	1.5	6.0	3.2	5.1	2.7	22.5	1.6	4.3	3.5
7	5.3	2.9	19.1	1.6	4.8	2.9	5.3	2.7	24.0	1.4	4.0	3.8
9	5.9	2.9	29.4	1.8	-	-	5.7	3.0	26.9	1.3	5.2	3.3
11	5.6	3.6	12.9	1.5	-	-	5.4	2.9	23.2	1.5	4.1	3.6
V208												
1	6.1	2.8	14.8	1.6	7.3	3.2	5.8	2.9	41.8	1.6	6.5	2.8
3	5.9	2.9	16.9	1.6	6.8	2.9	5.3	3.0	41.1	1.5	-	-
5	6.1	2.9	28.6	1.8	-	-	5.6	3.1	42.9	1.5	4.6	2.7
7	6.3	2.9	30.5	1.9	-	-	5.4	2.9	42.9	1.5	3.8	2.2
9	6.1	2.9	14.2	1.6	7.0	2.8	5.5	2.8	52.4	1.4	4.5	2.3
11	6.4	2.8	11.0	1.5	7.4	3.2	5.2	2.9	41.8	1.5	5.8	2.8
V234												
1	5.8	2.8	14.3	1.4	5.2	3.0	5.4	2.8	29.7	1.6	6.8	3.1
3	6.3	3.2	16.2	2.0	4.4	2.3	5.8	2.9	35.3	1.5	4.9	2.3
5	5.9	2.8	18.2	2.1	4.2	2.2	5.3	2.8	33.4	1.6	7.3	2.7
7	6.3	3.1	10.7	1.6	4.9	2.8	5.5	2.7	34.7	1.5	4.5	2.2
9	6.3	3.2	15.8	1.8	4.4	2.6	5.8	3.1	39.8	1.4	4.9	2.0
11	6.1	2.9	11.1	1.7	5.7	3.1	5.6	2.8	33.6	1.6	5.0	3.0
LSD												
Isol.	0.09	0.15	1.67	0.08	0.13	0.09	0.08	0.07	4.21	0.05	0.49	0.10
Sub-cult.	0.13	0.21	2.38	0.11	0.19	0.13	0.11	0.10	5.95	0.08	0.69	0.17
Isol*Subcul.	0.22	0.37	4.13	0.18	0.33	0.23	0.20	0.19	10.30	0.13	1.20	0.22

[a]and [b] – values are means of length (L), µm and width (W), a = mean length (µm) and b = mean width (µm) measured using light microscopy and image analysis, n = 50.

through which the fungus was passaged ($F_{5,315}$=14.83, P<0.001). The total number of conidia that adhered to aphid cuticle after 24 h incubation was also subject to three way (isolate-media-sub-culture) interaction (($F_{10,315}$=4.30, P<0.001).

Attenuated sub-cultures of each isolate (sub-culture 9 of V245, sub-cultures 3, 7 and 9 of V208 and sub-cultures 3 and 5 of V234) were observed to have consistently bound more conidia than those of non-attenuated cultures. Attenuated conidia, however, appeared to germinate slower and formed very few, if any, appressoria on aphid cuticles compared with non-attenuated (Table 3.4) conidia (Plates 3.3 and 3.4). Conidia that had been sub-cultured on SDA medium and incubated on insect dorsal abdomens for 24 h, appeared longer and wider in shape (5.96 μm X 2.99 μm) (Table 3.5 and Plate 3.3 a-c) than conidia grown on MM medium (5.57 μm X 2.85 μm) (Plate 3.3 d-e). Conversely, conidia harvested from SDA-grown colonies and which had germinated within 24 hours, were noted to have produced significantly shorter and wider germ-tubes (Table 3.5 and Plate 3.4 a-d) than those from MM-grown inocula (Table 3.5 and Plate 3.4 e-f).

Successive sub-culturing on both SDA and MM media significantly affected the dimensions of germlings (Table 3.5) for all three isolates tested (p<0.001). For example, conidia of sub-cultures 3 and 9 of V245, produced longer germ-tubes (19.1μm and 29.4 μm, respectively) than those of non-attenuated sub-cultures 1and 5 (12.0 μm and 12.1 μm, respectively). Sub-culturing was also observed to influence the size of appressoria produced (P<0.001) with non-attenuated cultures forming bigger appressoria (Plate 3.4 f) than those of attenuated cultures (Plate 3.4 e).

Plate 3.3a-e. Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V208 on aphid cuticle following incubation for 24 h at 23 °C (fluorescence microscopy, x1000): **a.** a conidium of sub-culture 1 from SDA produced a long and thick appressorium on the end of relatively short germ-tube; **b.** similar to that of a. conidia of sub-culture 5 from SDA formed large appressoria; **c.** a great number of conidia of sub-culture 9 adhered to the aphid cuticles; **d.** conidia of sub-culture 1 passaged through MM produced appressoria on the end of a long germ-tube; **e.** conidium of sub-culture 9 passaged through MM produced significantly longer and thinner germtubes than that in d. SDA = Sabouraud Dextrose Agar, MM = Minimal Medium. Bar = 10 μm.

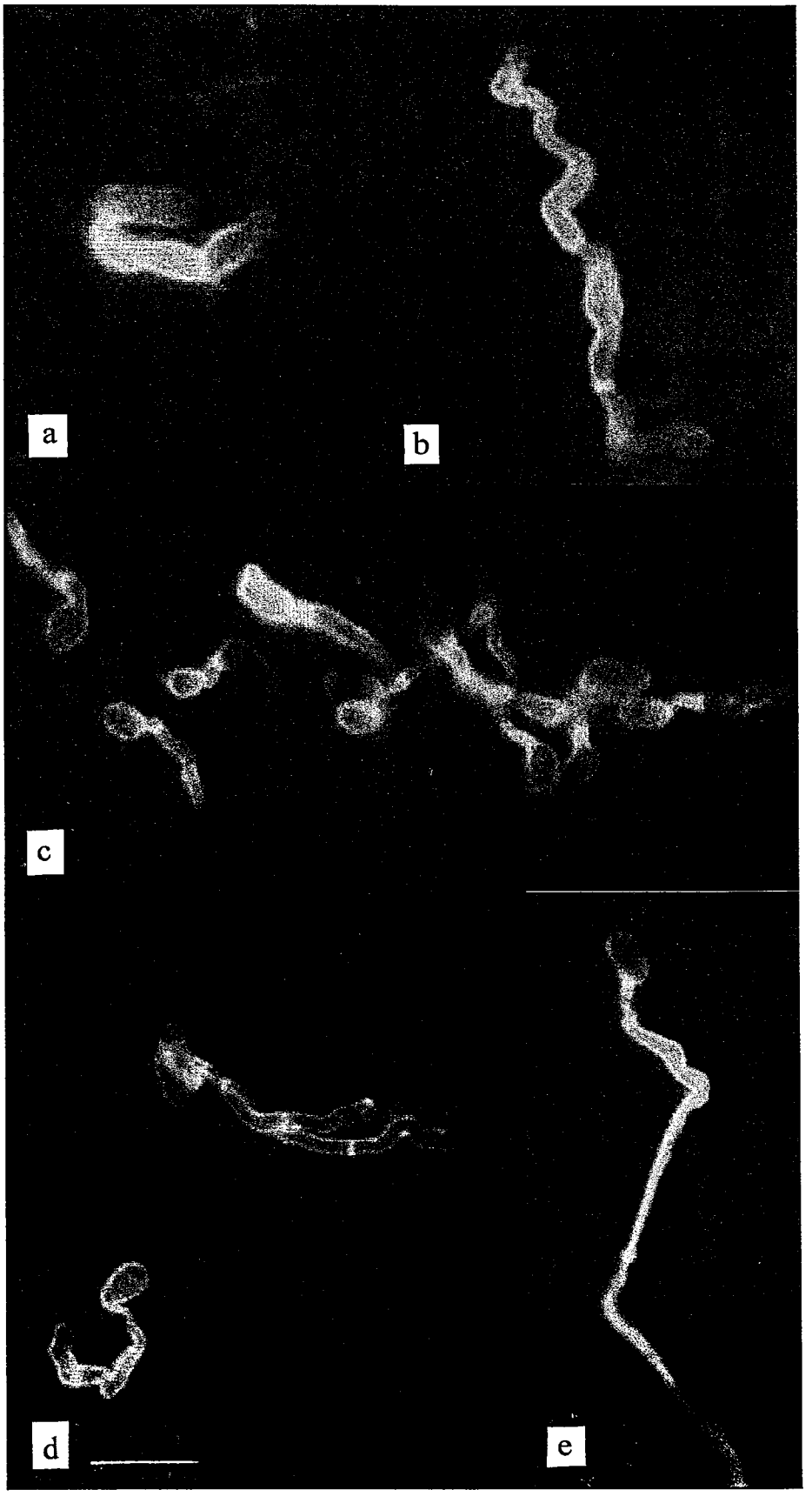
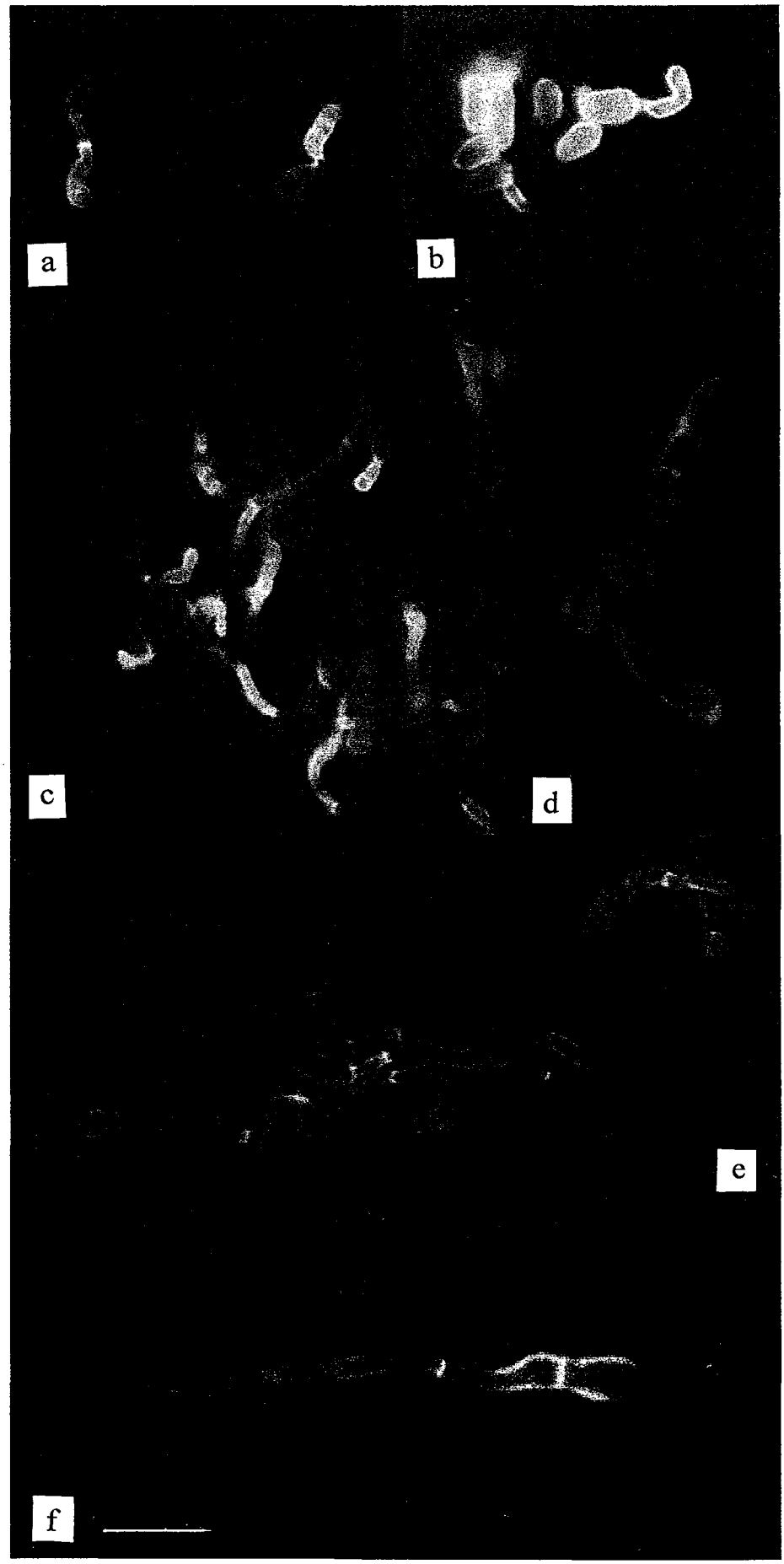

a
b
c
d
e

Plate 3.4a-f. Effect of successive sub-culturing on germination behaviour of *Metarhizium anisopliae* isolate V234 on aphid cuticle following incubation for 24 h at 23 °C (fluorescence microscopy, x1000): **a.** conidia of sub-culture 1 passaged through SDA; **b.** greater number of attenuated conidia (sub-culture 3 from SDA) attached to aphid cuticle in comparison to sub-culture 1, however, very few germinated and produced appressoria; **c.** similar to sub-culture 3 germination of attenuated sub-culture 5 is slow with smaller number of germlings producing appressoria; **d.** a large number of conidia of sub-culture 11 passaged through SDA produced large appressoria; **e.** conidia of sub-culture 9 passaged through MM produced considerably longer germtubes than that in c. and formed small appressoria on the end of a long germtube (note penetration pegs (arrows)); **f.** more aggressive conidium produced significantly larger appressorium than that of less aggressive conidia in e. SDA = Sabouraud Dextrose Agar, MM = Minimal Medium. Bar = 10 μm.


a
b
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f

3.3.4 Mortality and mean survival times of *M. persicae* infected with *M. anisopliae.*

Results in Table 3.6 show that the first sub-culture of all three isolates grown on SDA was highly virulent to aphids causing 60%-88% mortality within seven days. After only 3 sub-cultures the pathogenicity of the fungus rapidly declined resulting in longer survival time ($F_{10,1979}$=51.77) and markedly reduced mortality ($F_{10,132}$=11.77) (Table 3.6). Higher levels of virulence occurred in sub-culture 5 (V245), 11 (V208) and 7 (V234) in the absence of passaging through an insect. However, although inoculum from sub-cultures 7 (V245) and 5 (V234), when tested against aphids, resulted in high mortality (72% and 78%, respectively), the time taken to kill 50% insects (LT_{50} value) was significantly longer (6.6 days and 6.3 days, respectively) ($P<0.001$) than it was for more virulent sub-cultures (Table 3.6). Conidia induced from sterile mycelium were just as virulent as conidia from sporulating cultures sub-cultured at the same time.

All sub-cultures of all three isolates passaged through nutrient-depleted media (MM) were more pathogenic against aphids, causing 58%-92% mortalities (Table 3.6), than the same conidia from SDA sub-cultures. The virulence of inocula, as measured by LT_{50}, at the start of serial passages was 4.8 days for V245, 5.3 days for V208 and 4.9 days for V234, respectively. Sub-culturing the fungus on MM significantly lowered LT_{50} values in almost all successive sub-cultures of isolate V245. However, these values increased ($P<0.001$) by more than 0.5 and 1.0 days for sub-cultures 3 and 7, respectively, for V208 isolate and by 0.4 and 0.7 days for sub-cultures 7 and 9, respectively, for isolate V234 (Table 3.6).

Table 3.6. Effect of sub-culturing three isolates of *Metarhizium anisopliae* on SDA and MM artificial media on mortality and lethal time (LT_{50}) against *Myzus persicae* following 7 days incubation at 23°C.

Sub-culture	V245				V208				V234			
	Mortality [a] (%)		LT_{50} [b] (days)		Mortality (%)		LT_{50} (days)		Mortality (%)		LT_{50} (days)	
	SDA	MM	SDA	MM	SDA	MM	SDA	MM	SDA	MM	SDA	MM
1	80 (2.3)	85 (3.4)	5.1 (0.6)	4.8 (1.3)	60 (3.1)	73 (2.2)	6.0 (0.8)	5.3 (0.9)	68 (1.8)	88 (1.5)	5.8 (1.1)	4.9 (0
3	76 (2.8)	89 (3.0)	5.9 (1.1)	4.1 (1.2)	38 (3.7)	64 (2.9)	6.8 (0.5)	5.8 (0.7)	47 (3.4)	74 (2.0)	6.2 (1.2)	5.2 (0
5	82 (2.6)	92 (1.9)	5.3 (1.5)	3.9 (0.9)	56 (2.3)	78 (2.1)	6.2 (0.4)	5.0 (1.1)	44 (3.1)	78 (1.9)	6.3 (0.9)	5.0 (0
7	72 (2.9)	87 (2.5)	6.6 (0.7)	4.0 (1.0)	28 (3.9)	58 (3.0)	6.9 (1.0)	6.3 (0.4)	72 (1.7)	72 (1.9)	5.6 (1.1)	5.3 (1
9	52 (3.3)	70 (3.1)	6.9 (0.4)	4.5 (1.1)	49 (3.5)	70 (2.5)	6.2 (0.6)	5.5 (0.6)	67 (1.9)	66 (2.1)	5.8 (0.7)	5.6 (1
11	70 (2.2)	77 (2.4)	6.0 (0.7)	4.0 (1.0)	71 (2.8)	83 (2.3)	5.8 (0.4)	5.2 (0.4)	70 (1.9)	78 (1.8)	5.7 (1.2)	5.1 (0

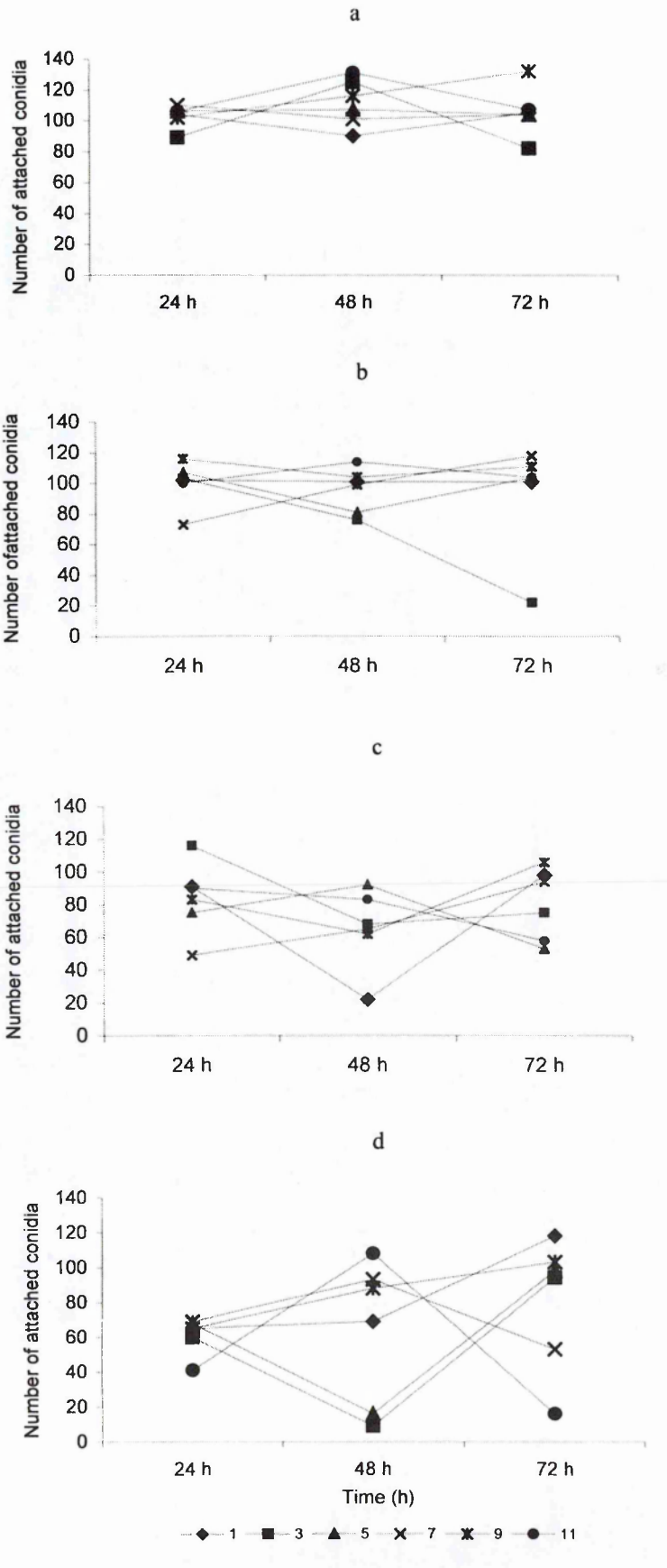
[a] Total mortality 7 days post-inoculation. Number in parentheses are standard errors.
[b] LT_{50} = time in days when 50% of *M. persicae* individuals are dead. Numbers in parentheses are standard deviations.

Mortality in control samples averaged less than 3% during the entire study.

3.3.5 The adhesion and germination behaviour of the three *M. anisopliae* isolates on artificial surface

To compare the adhesion and germination behaviour of attenuated and non-attenuated inocula on a polystyrene surface, conidia of respective sub-cultures of isolate V245 were suspended in water, glucose, yeast extract or both, and then incubated on artificial surface for 72 h. Twenty four hours post-incubation, all sub-cultures of *M. anisopliae* firmly attached to polystyrene (Fig. 3.2a-d). However, the total number of conidia that remained attached to the surface depended significantly on the solution the fungus was suspended with, the incubation time, and the ability of each sub-culture to adhere to polystyrene ($F_{30,284}$=33.52, P<0.001). When suspended in distilled water (Fig.3.2 a), many conidia adhered to polystyrene for almost all sub-cultures with the exception of sub-culture 3, where significantly fewer (approximately 80) conidia remained attached to the artificial surface after 72 h post-incubation. Suspension of conidia in glucose greatly reduced the initial 24 h incubation attachment of conidia for sub-culture 7, however, the total number of conidia remaining attached to polystyrene after 72 h incubation period, significantly increased from 73 to 118 conidia (Fig 3.2 b). In contrast, adhesion of sub-culture 3 dramatically fell from 103 to only 22 with increasing time of incubation (Fig 3.2 b). Similar results were also observed when conidia were suspended in yeast extract, although adhesion was generally lower than that observed for water or glucose treatments (Fig.3.2 c). However, adhesion behaviour of conidia from each sub-culture tested was significantly different when suspended in glucose-yeast-extract solution (Fig. 3.2 d). For example, the initial 24 h incubation time, resulted

Figure 3.2a-d. Effect of sub-culturing on SDA on the number of V245 conidia adhering to a polystyrene surface following incubation for 24, 48 and 72 h at 23° C in: distilled water (a), 1% (w/v) glucose (b), 1% (w/v) yeast extract (c) and combination of 1% glucose and 1% yeast extract (d). LSD (Med*Sub-cult*Time) = 11.9, $P < 0.001$.

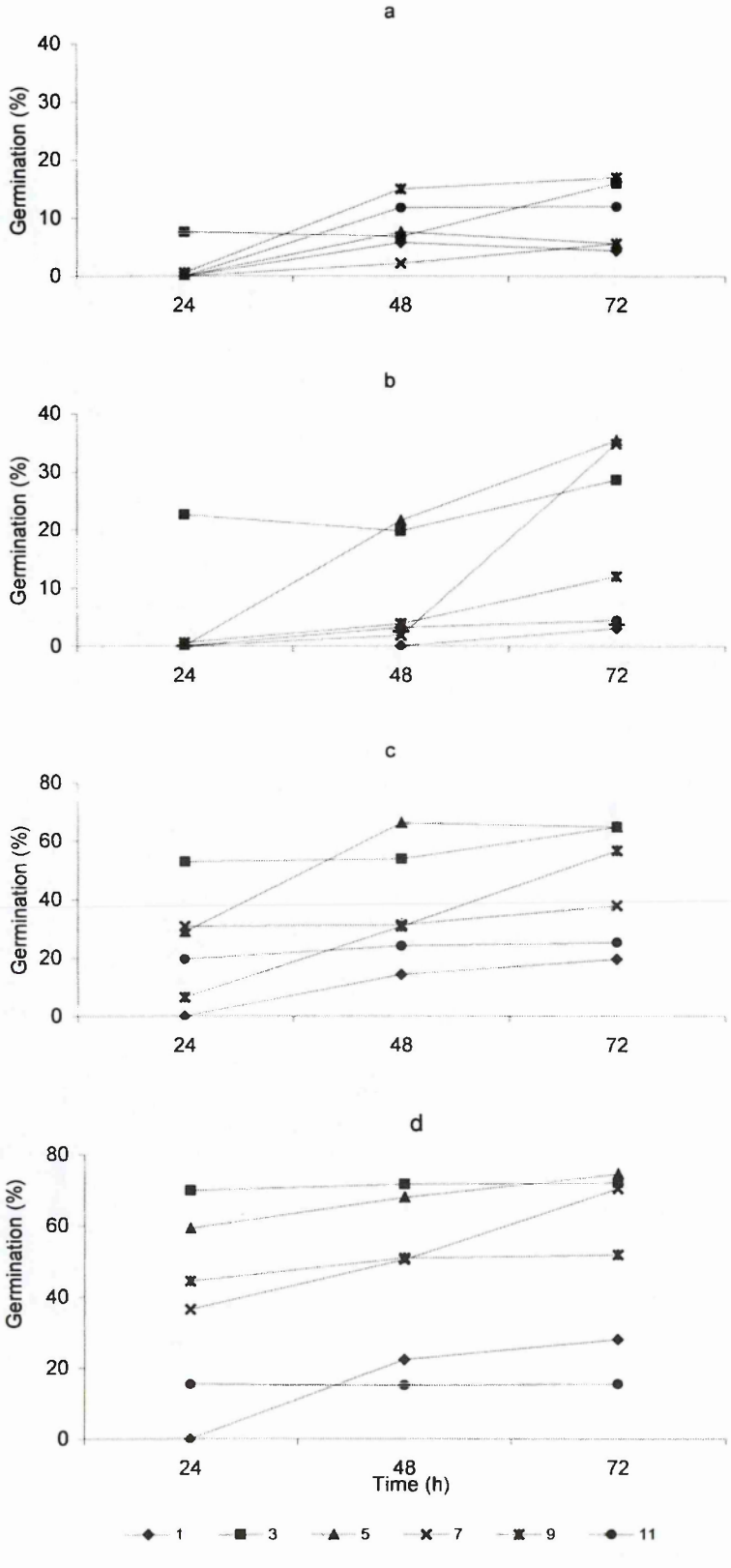

a
Number of attached conidia
140
120
100
80
60
40
20
0
24 h
48 h
72 h
b
Number ofattached conidia
140
120
100
80
60
40
20
0
24 h
48 h
72 h
c
Number of attached conidia
140
120
100
80
60
40
20
0
24 h
48 h
72 h
d
Number of attached conidia
140
120
100
80
60
40
20
0
24 h
48 h
72 h
Time (h)
1
3
5
7
9
11
Sub-culture number

in 40 conidia of sub-culture 11 and between 60-70 conidia of sub-cultures 1, 3, 5, 7 and 9 adhering to the polystyrene surface. An additional 48 h incubation period, resulted in a significant reduction of conidia for sub-culture 11 and 7, and considerable increase of attachment to the artificial surface for sub-cultures 1, 3, 5 and 9 (Fig. 3.2 d).

The germination behaviour of all sub-cultures tested is presented in Figure 3.3. Generally, very few or no conidia of isolate V245 germinated within the first 24 hours when suspended in distilled water (Fig. 3.3a-d and Plate 3.5a-f). At 48 (Plate 3.6a-f) and 72 h inculation, however, germination for all sub-cultures significantly increased (Fig. 3.3a-d). After 24 h incubation in 1% (w/v) glucose, conidia of sub-cultures 1,5, 7 and 11 failed to germinate (Fig. 3.3a-d), compared to more than 20% of conidial germination (Plate 3.7 a) for sub-culture 3. A spontaneous mycelial development (rapid growth and branching) was observed for sub-culture 5 after 48 h in 1 % glucose solution. Following 72 h suspension, all sub-cultures of isolate V245 developed long branching hyphae. In addition, sub-culture 3 was observed to produce a large number of submerged conidia (Plate 3.8a), whereas sub-culture 5 was recorded to form a great number of appressoria of different size and shape (Plate 3.8b) suggesting that only these two sub-cultures were able to switch from trophic growth to reproductive differentiation.

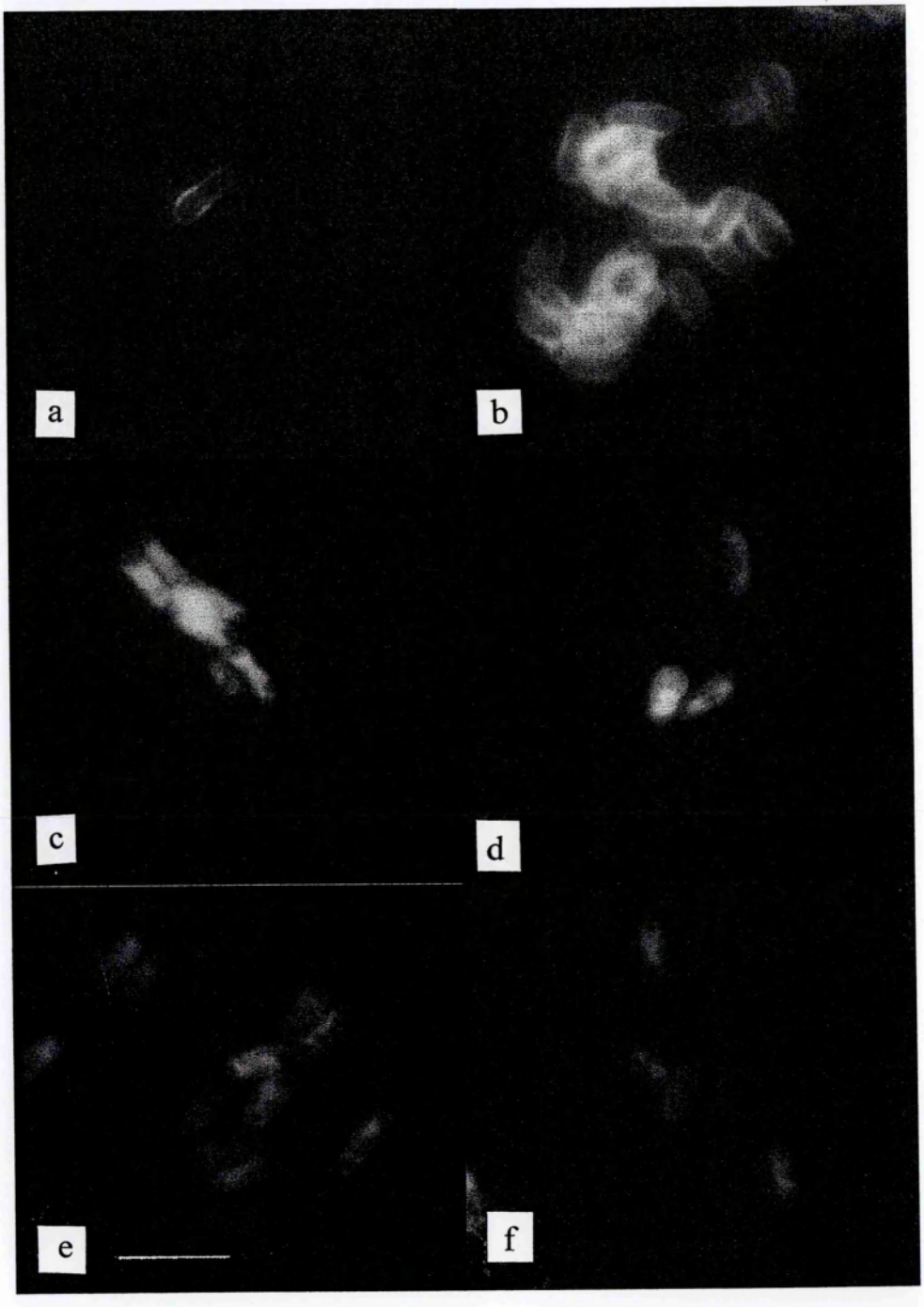
A significantly higher conidial germination, ranging from 20% to 55%, was observed for conidia of sub-cultures 11, 5, 7 and 3 suspended in yeast extract after 24 h suspension time (Plate 3.9a-d). However, germ-tube development was very similar to

Figure 3.3a-d. Effect of sub-culturing on SDA on germination of V245 conidia following incubation for 24, 48 and 72 h at 23° C in distilled water, 1% (w/v) glucose, 1% (w/v) yeast extract and combination of 1% glucose and 1% yeast extract. Bars are standard errors of means.



Sub-culture number

Plate 3.5a-f. Effect of successive sub-culturing on SDA on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 24 h at 23 °C in distilled water (fluorescence microscopy, x1000): **a.** sub-culture 1; **b.** sub-culture 3; **c.** sub-culture 5; **d.** sub-culture 7; **e.** sub-culture 9; **f.** sub-culture 11. Note that although there was no germination observed in any of the tested sub-cultures, there was a clear difference in the intensity of calcoflour fluorescence. Bar = 10 μm.


a
b
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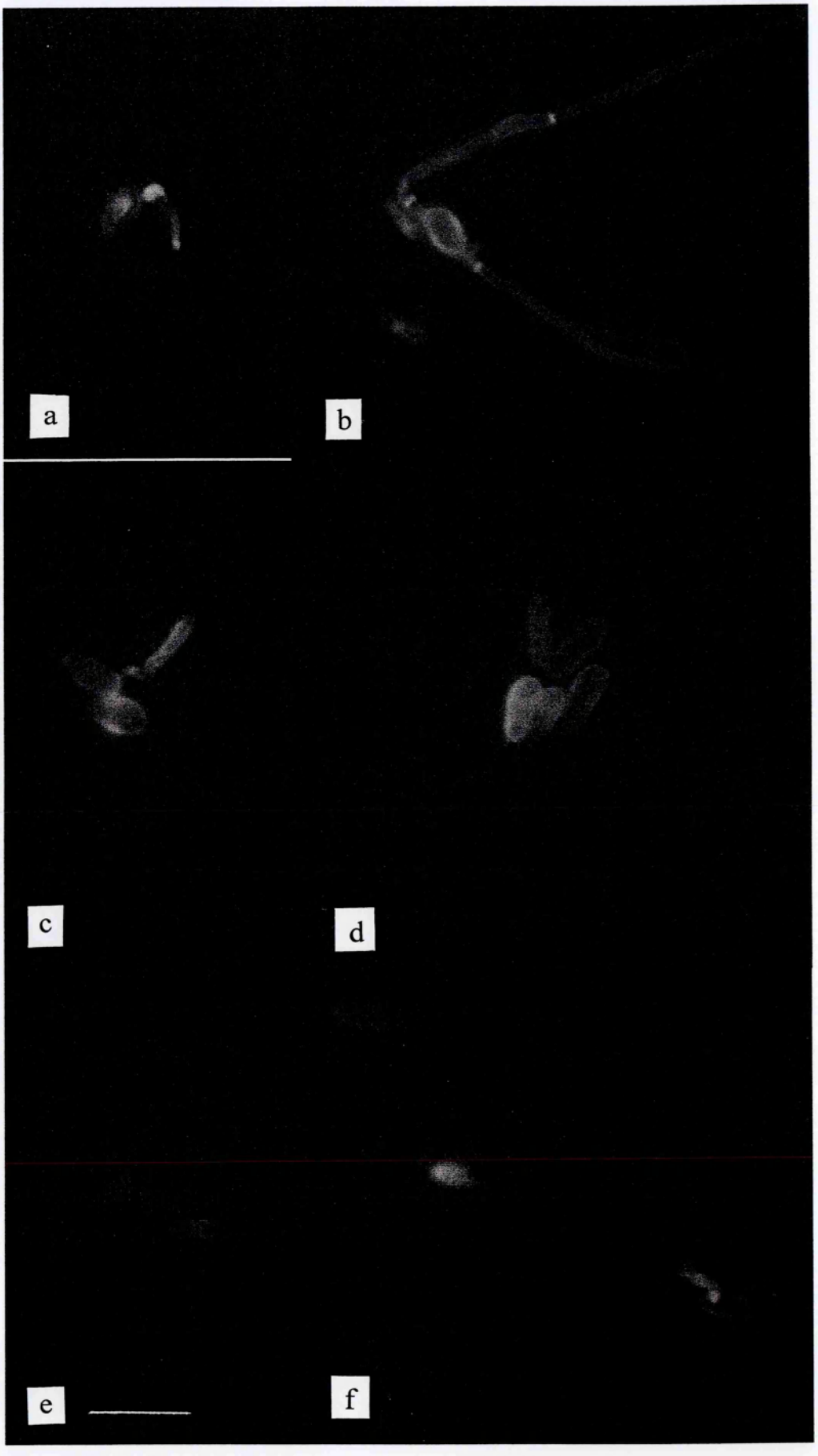
d
e
f

Plate 3.6a-f. Effect of successive sub-culturing on SDA on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 48 h at 23 °C in distilled water (fluorescence microscopy, x1000): **a.** sub-culture 1; **b.** sub-culture 3; **c.** sub-culture 5; **d.** sub-culture 7; **e.** sub-culture 9; **f.** sub-culture 11. Note that although germination of conidia occurred in all sub-cultures, the most advanced mycelial development was recorded for sub-culture 3. Bar = 10 μm.


a
b
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d
e
f

Plate 3.7a-d. Effect of successive sub-culturing on SDA on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation at 23 °C in glucose (fluorescence microscopy, x1000): **a.** sub-culture 9 for 24 h; **b.** sub-culture 3 for 48 h; **c.** sub-culture 5 for 48 h; **d.** sub-culture 9 for 48 h. Note a spontaneous mycelial development in sub-culture 5. Bar = 10 μm.


a
b
c
d

Plate 3.8a-c. Effect of successive sub-culturing on germination on SDA behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 72 h at 23 °C in glucose (fluorescence microscopy, x1000): **a.** sub-culture 3; **b.** sub-culture 5 **c.** sub-culture 11. Note that only sub-culture 3 produced a large number of submerged conidia, whereas sub-culture 5 formed a great number of appressoria of different shape and size. In contrast, a poorly developed germtube was typical to sub-culture 11. Bar = 10 μm.

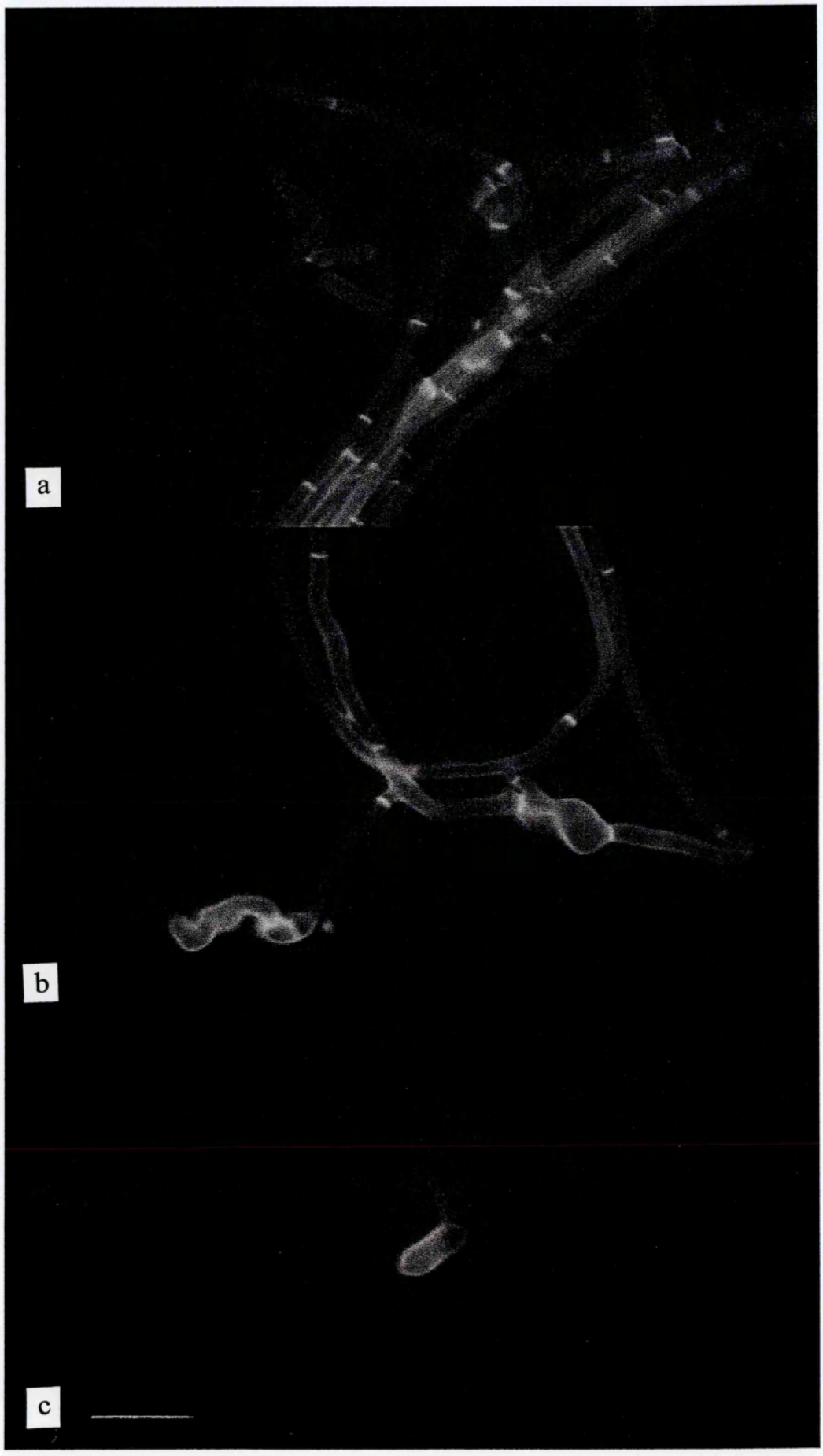
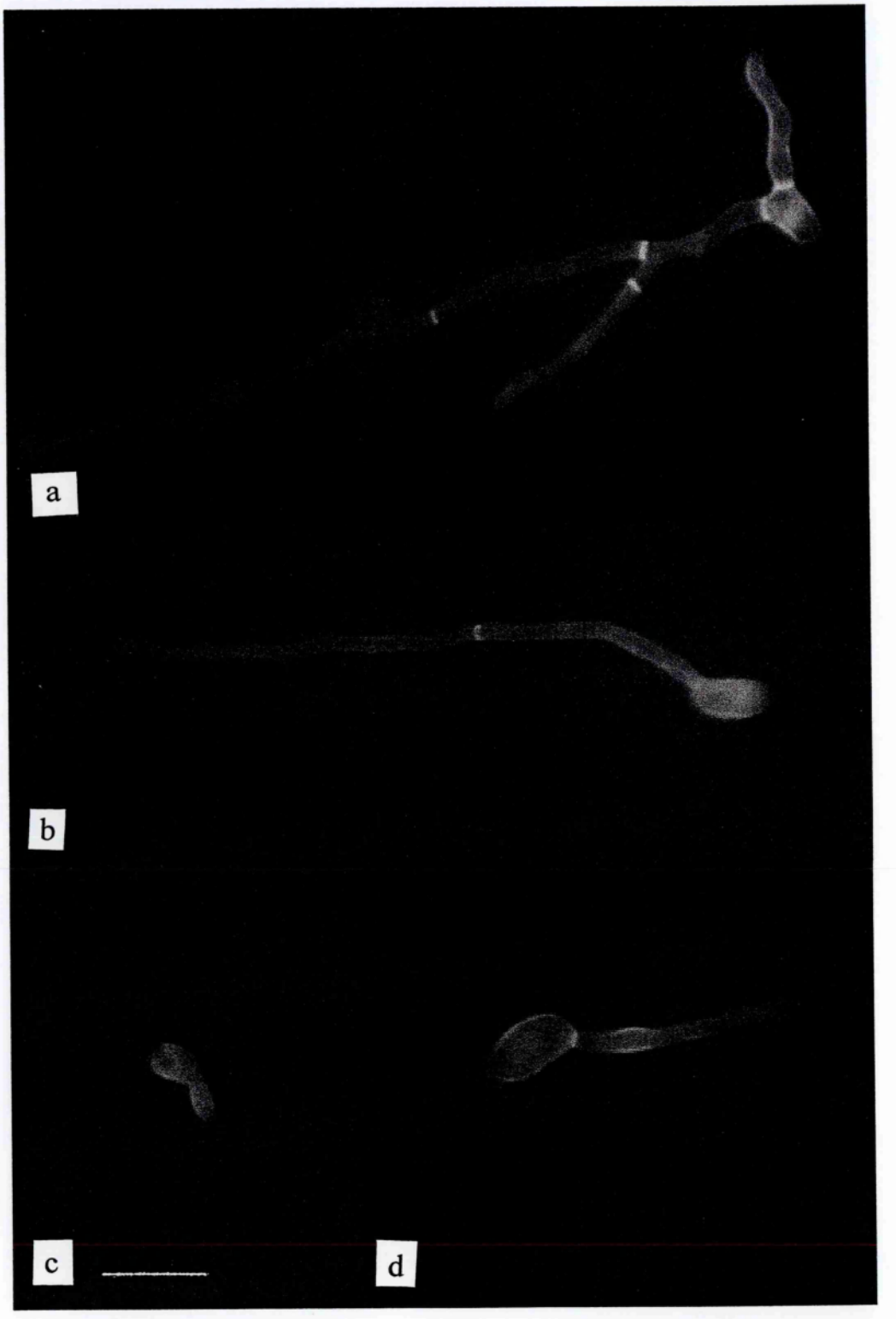
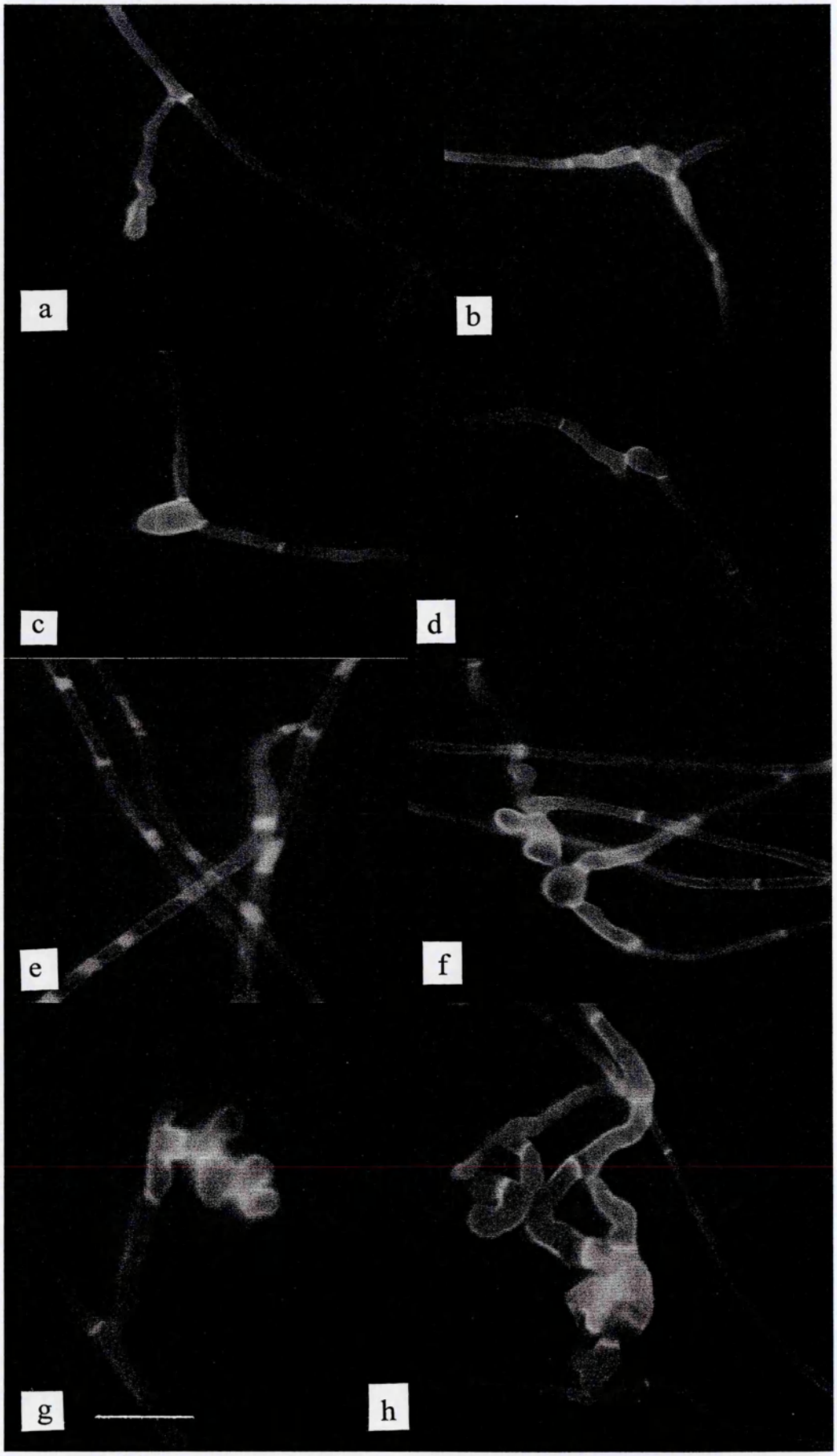

a
b
c

Plate 3.9a-d. Effect of successive sub-culturing on SDA on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 24 h at 23 °C in yeast extract (fluorescence microscopy, x1000): **a.** sub-culture 3; **b.** sub-culture 7; **c.** sub-culture 9; **d.** sub-culture 11. Note advanced germination and mycelial branching was characteristic to sub-culture 3 only. Bar = 10 μm.


a
b
c
d

that observed in glucose (Fig. 3.3a-d). A typical di- or tri-polar hyphal development was also noted for sub-cultures 7, 9 and 11 48 h post-incubation in yeast extract (Plate 3.10 b-d). In addition, sub-culture 5 was the only sub-culture forming a large number of simple appressoria alongside thin hyphae (Plate 3.10 a). Following 72 h incubation period in yeast extract, sub-cultures 3, 5 and 9 also produced a number of simple and complex appressoria (Plate 3.10 f-h). Suspending conidia in glucose-yeast-extract solution for 24 h, 48 h and 72 h had not only significantly improved conidial germination of all sub-cultures but also stimulated more extensive hyphal development and appressorial formation (Plates 3.11a-g and 3.12a-b) than for those sub-cultures suspended in water, glucose or yeast extract.

Plate 3.10a-h. Effect of successive sub-culturing on SDA on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 48 h (**a-d**) and 72 h (**e-h**) at 23 °C in yeast extract (fluorescence microscopy, x1000): **a.** sub-culture 5; **b.** sub-culture 7; **c.** sub-culture 9; **d.** sub-culture 11; **e.** sub-culture 1; **f.** sub-culture 3; **g.** sub-culture 5; **h.** sub-culture 9. Note that yeast extract induced appressorial formation after 48 h incubation for sub-culture 5 only. Distinctive dipolar germination behaviour was recoded for sub-cultures 7, 9 and 11. Following 72 h incubation period a large number of simple and complex appressoria were developed in sub-cultures 3, 5 and 9. Bar = 10 μm.


a
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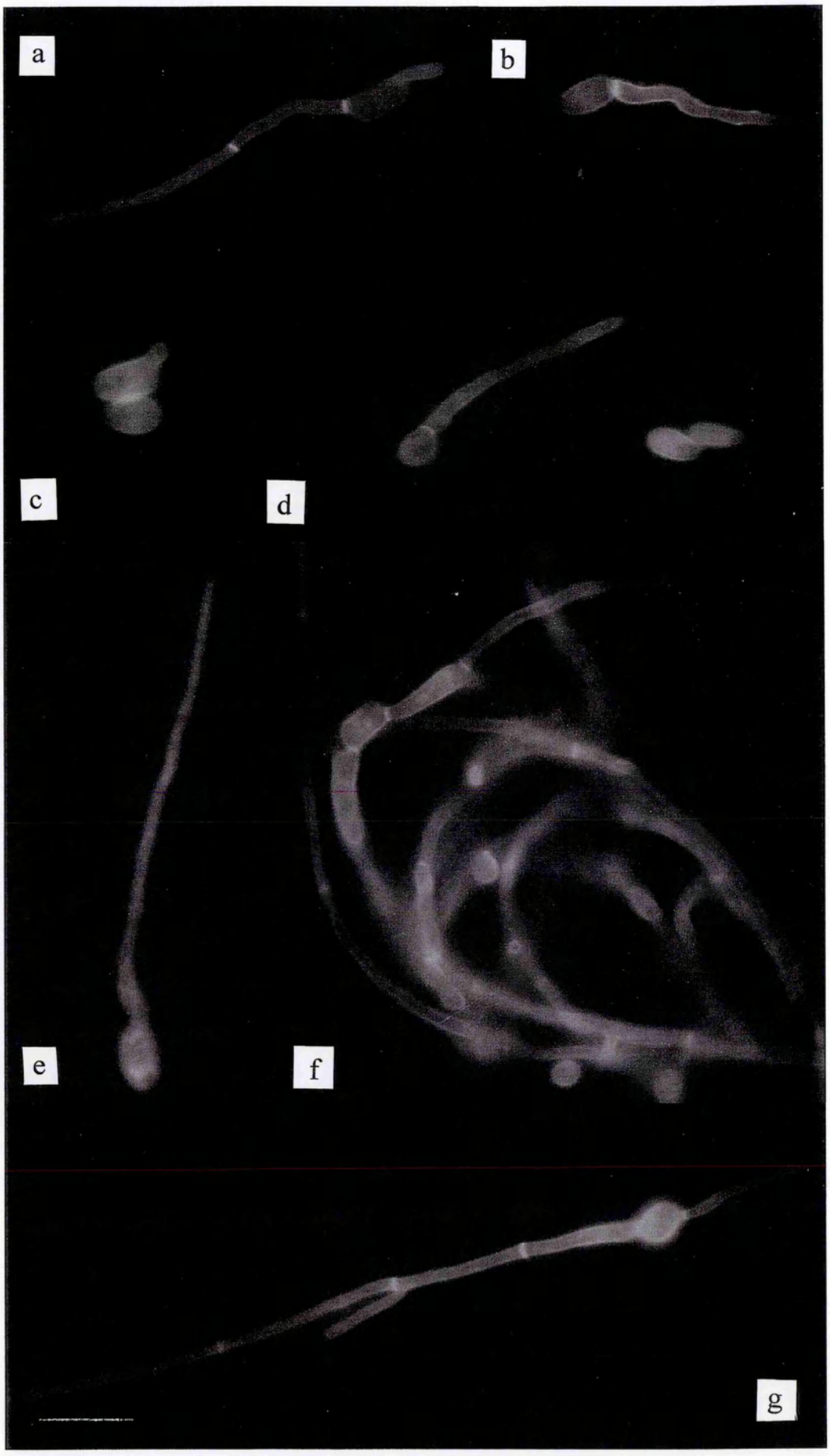
e
f
g
h

Plate 3.11a-g. Effect of successive sub-culturing on SDA on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 24 h (**a-d**) and 48 h (**e-g**) at 23 °C in yeast extract and glucose (fluorescence microscopy, x1000): **a.** sub-culture 3; **b.** sub-culture 5; **c.** sub-culture 7; **d.** sub-culture 9; **e.** sub-culture 1; **f.** sub-culture 7; **g.** sub-culture 9. Note that addition of glucose to yeast extract activated spore germination for almost all sub-cultures tested suppressing extensive hyphal growth in sub-culture 7 and stimulating the growth in sub-culture 9 after 24 h incubation. However, after 48 h incubation period a spontaneous elongation of hyphae was observed in sub-culture 7 only. Bar = 10 μm.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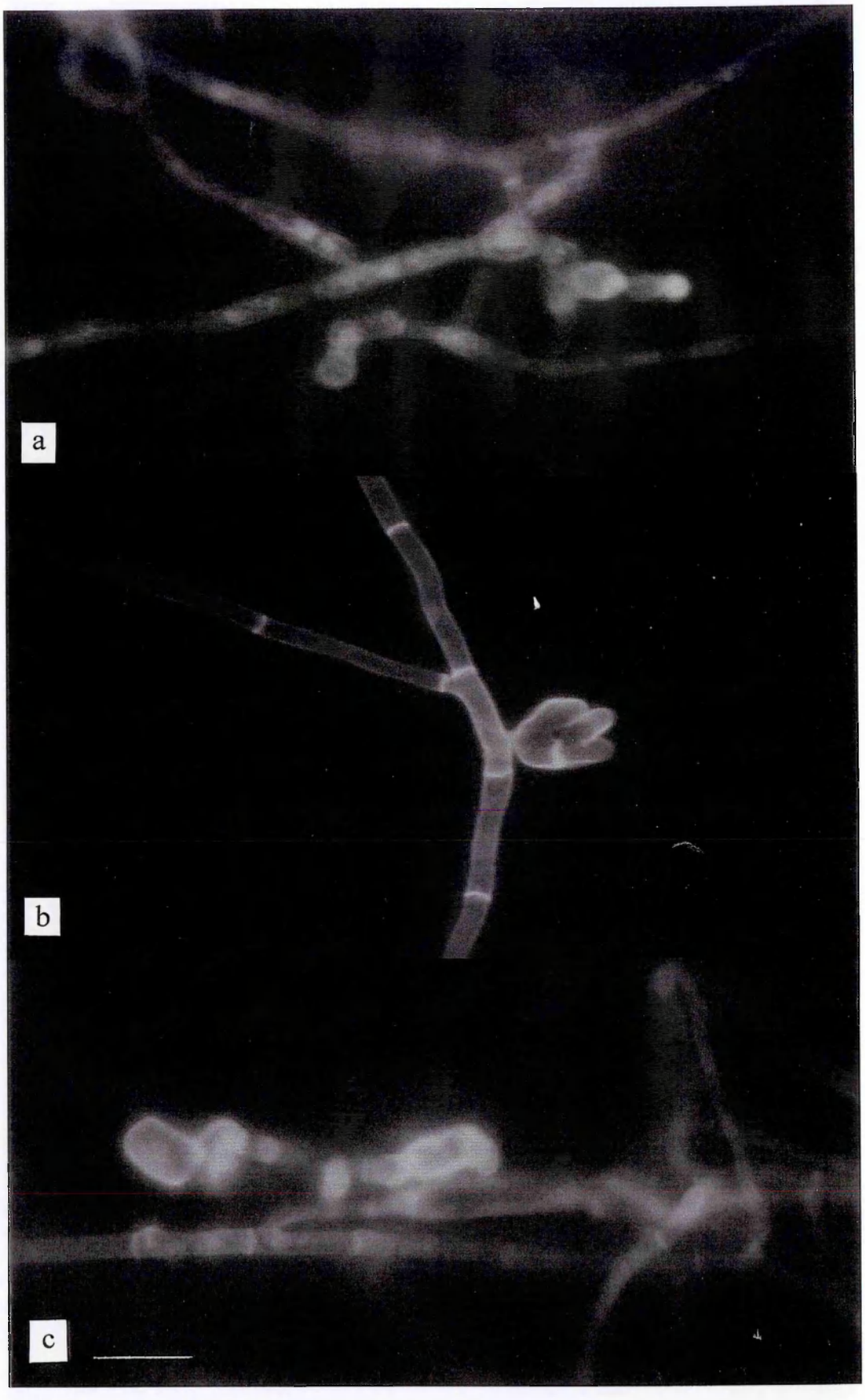
g

Plate 3.12a-c. Effect of successive sub-culturing on SDA on germination behaviour of *Metarhizium anisopliae* isolate V245 on polystyrene following incubation for 72 h at 23 °C in yeast extract and glucose (fluorescence microscopy, x1000): **a.** sub-culture 3; **b.** sub-culture 7; **c.** sub-culture 9. Note extensive hyphal development for sub-cultures 3, 7 and 9. Only sub-culture 7 produced large, often branching, appressoria. Bar = 10 μm.


a
b
c

3.4 DISCUSSION

Parental cultures of *M. anisopliae* usually germinate, grow and sporulate well on Sabouraud Dextrose Agar. However, this study clearly demonstrates that when isolates of this pathogen are repeatedly sub-cultured on the nutrient-rich medium over time, their growth and development are significantly affected. Serial *in vitro* sub-culturing has been shown to reduce conidial germination, fungal growth rate, amount of aerial mycelium and spore production in addition to inducing changes in colony colour of many entomopathogenic fungi including *S. pracina, I. fumosorosea, O. destructor, A. flavus, A. oryzae, B. bassiana* (Kawakami, 1960), *V. lecanii* (Hall, 1980), *N. rileyi* (Lord & Roberts, 1986; Morrow *et al.,* 1989) and *E. neoaphidis* (Wilding *et al.,* 1992). For example, Hall (1980) demonstrated a significant reduction in growth rates and changes in colony morphology (reduction in mycelial production and changes of colony colour) for two *V. lecanii* isolates when successively sub-cultured more than 50 times on SDA.

A significant decrease in spore production and the appearance of sterile colonies as a result of repeated sub-culturing, were also reported for the aphid-pathogenic fungus *E. neoaphidis* (Wilding *et al.,* 1992). Such observations are in agreement with the findings reported in this study, where sub-culturing of *M. anisopliae* isolates three times on SDA, was shown to significantly reduce sporulation and conidial germination, influence the fungal growth and colour of fungal colonies and induce sectors of sterile colonies. However, isolate V208 appeared to be more sensitive to repeated sub-culturing and became sterile much more readily than isolate V245. In contrast, isolate V245 consistently sporulated well, producing large quantities of dark-green conidia.

In contrast to sub-culturing on SDA, eleven successive sub-cultures on MM media had no effect on morphology or phenotype of all three isolates of *M. anisopliae* tested. Artificial medium has been shown to have a significant influence on colony morphology of other entomopathogenic fungal species (Hall, 1980, Lord & Roberts, 1986). For example, Hall (1980) observed that repeated sub-culturing of a *V. lecanii* strain on SDA or Potato Dextrose agar (PDA), produced more than two morphological variants, compared to sub-culturing on Malt extract or Czapek-Dox agars, where caused no morphological or phenotypic changes were observed.

Sterile mycelium was shown to be encouraged to produce conidia by simply damaging the hyphae, however, specific reasons why this should be are not clear. Stress or compounds released from damaged hyphae may provide stimulus, which encourage conidial production, however, further studies are required in order to determine the precise mechanism responsible. The fact that conidia, produced from mechanically damaged sterile hyphae, were pathogenic, suggests that factors affecting the morphology may operate independently to those influencing pathogenicity. This assumption is supported by the findings of Hall (1980), who described alteration in the morphology of *V. lecanii* colonies after repeated sub-culturing on SDA or PDA, but noted no parallel changes in virulence against aphid hosts.

Continuous culturing *in vitro* of the parasitic protozoa *Cryptobia salmositica,* which causes salmonid cryptobiosis in salmon, resulted in an attenuation of its virulence (Woo & Li, 1990) together with differences in protein profile (Woo & Thomas, 1991). In this study, changes in virulence, together with differences in non-specific esterase profile, was recorded for inocula of *M. anisopliae* during successive sub-culturing on both SDA

and MM media. Attenuation of virulence, as measured by LT_{50}, was manifested in an interesting way, with LT_{50} values increasing after just three successive sub-cultures before rapidly decreasing (V234) or steadily increasing after 5 sub-cultures (V245), or with LT_{50} values abruptly recovering after sub-culture 11 (V208) (Table 3.6). Most studies to date report a gradual or rapid decline in virulence with recovery only being achieved after passaging through an insect host (Hajek *et al.*, 1990; Butt & Goettel, 2000). In this study, recovery of virulence was shown to occur even in the absence of passaging through a host insect. It is possible, however, that had sub-culturing continued beyond the 11 successive sub-cultures used in this study, a more marked decline in spore production may have been observed, possibly leading to the production of sterile cultures with complete loss of pathogenicity.

It is unlikely that bioassays alone would reveal the full variation in expression of attenuation. However, in combination with studies on physiological behaviour of infective propagules, further information could be gathered about the attributes of virulence. For example, rapid germination has been previously attributed to virulent strains (Al-Aidroos & Seifert, 1980; Altre *et al.*, 1999). Indeed, in this study, conidia of *M. anisopliae* isolates cultured on MM germinated considerably faster, formed appressoria more frequently, and incited faster infection and rapid kill of aphids than conidia cultured on SDA. Nutritional conditions are known to alter composition and attributes of many fungal pathogens (Lane *et al.*, 1991a, 1991b; Jackson & Schisler, 1992; Hallsworth and Magan, 1994a, 1994b, 1994c 1995; Ibrahim *et al.*, 2002) and thus influence physiological behaviour. For example, Lane *et al.* (1991a) demonstrated a significant increase in accumulation of endogenous lipids and carbohydrates in blastospores of *B. bassiana* produced in nitrogen-limited media, whereas conidia

produced on a medium with a low carbon/nitrogen ratio was shown to accumulate more endogenous protein and lipids (Schisler *et al.*, 1991; Jackson & Schisler, 1992).

High concentrations of endogenous proteins in pathogenic inocula is known to provide the amino acid pool necessary for protein synthesis and thus facilitate rapid germination and frequent appressoria formation (St. Leger *et al*, 1989, Bhairi *et* al, 1990; Jackson & Schisler, 1992). Moreover, the addition of KCl to SDA media, has also been reported to affect surface carbohydrates such as β-glucans (Ibrahim *et al.*, 2002), which are known to influence adhesion of many micro-organisms to their respective hosts (Doyle & Taylor, 1994; van der Drift *et al.*, 1998; Price, 1999; Spaink, 2000; Ibrahim *et al.*, 2002). Repeated sub-culturing under the same nutritional and environmental conditions carried out in these studies, demonstrated a significant change in adhesion and germination behaviour of conidia of all three isolates of *M. anisopliae* tested. Furthermore, the number of conidia which adhered to aphid cuticles increased with successive sub-culturing. However, this resulted neither in an augmented mortality nor in speedy infection, suggesting that the fungus became attenuated. As such, inocula was slower to germinate and failed to produce appressoria. Conversely, for non-attenuated inocula, very few propagules were needed to initiate rapid infection and high aphid mortality. In addition, for those few conidia which successfully attached to insect cuticles, germination was faster and the proportion of germlings producing appressoria was higher when compared to attenuated conidia. Also, virulent inocula consistently produced shorter germ-tubes and larger appressoria than less virulent inocula.

The amount of nutrients available to fungus during infection has been shown to regulate appressorium differentiation (St Leger *et al.*, 1989; Magalhaes *et al.*, 1991b;

Butt *et al.*, 1995). For example, a large number of sporelings on flea beetle (*Psylliodes chrysocephala*) cuticle which contained low concentration of nutrients (Hunt *et al.*, 1984) produced appressoria, yet comparatively few did so on aphid cuticle where nutrients were mostly derived from honeydew (Butt *et al.*, 1995). Also, the morphology of produced appressoria has been shown to be influenced by cuticular surfaces (St Leger *et al.*, 1986; Butt *et al.*, 1995) For example, appressoria produced on aphid cuticle were smaller than those on beetle cuticles (Butt *et al.*, 1995) and appressoria produced in close proximity tended to be morphologically similar (Plate 3.4e; Butt *et al.*, 1995), suggesting that the pathogen receives similar cues at that site and that the cues differ from one site to the next.

Nutritional solutions such as glucose, yeast extract and a combination of both have also been shown to significantly influence attachment, germination and reproductive differentiation on polystyrene surface of attenuated and non-attenuated sub-cultures of *M. anisopliae* isolate V245 used in this study. Although fewer conidia of attenuated sub-culture 3 were observed to adhere to artificial surface after 72 h incubation in the solutions tested, such conidia were more and faster to germinate and produce appressoria, and submerged conidia than any of non-attenuated conidia. However, such observations are in contrast to those recorded during *in vivo* studies, where fewer conidia of attenuated sub-cultures attached to aphid cuticle, germinated slower and failed to produce appressoria on the cuticle of *Myzus persicae*. These observations support the hypothesis that infective propagules possess a number of physiological and biological provisions which allow to detect and respond to external stimuli and that this complex interaction between various pathways involved in virulence and morphogenesis can be disrupted by *in vitro* sub-culturing. Disruption of the N-

acetylglucosamine (GlcNAc) catabolic pathway gene cluster in *Candida albicans* has been shown to trigger high attenuation in virulence and significantly change the morphology including inability to grow on amino sugars and reduction in adherence to human buccal epithelial cells *in vitro* (Singh *et al.*, 2001). Indeed, when sub-cultures of *M. anisopliae* were checked for physiological response to glucose, yeast extract and the combination of both, they exhibited very different adhesion, germination, differentiation and growth behaviour on polystyrene surface suggesting that repeated *in vitro* sub-culturing might disturb the cell surface receptor(s) responsible for reception or transmission of signals.

In conclusion, this study shows that continuous sub-culturing of *M. anisopliae* isolates on synthetic media affects the phenotype, morphology and virulence of the fungus. The morphological changes such as reduction in sporulation and increased sterility become more prominent with each subsequent sub-culture on SDA media. Although there was no obvious link established between morphological changes and virulence, the attenuation of virulence was often associated with increased adhesion, slow germination and inhibition of appressorial differentiation on aphid cuticles. However, differences in appressorial morphology between attenuated and non-attenuated *Metarhizium* were shown to influence the speed of infection. Since successive sub-cultures of each isolate exhibited different response to nutritional solutions, as measured by morphological behaviour, it is possible that continuous sub-culturing alters conidial wall or conidial surface structure through polymerisation of GlcNAc into chitin, an integral component of the cell walls (Braun & Calderone, 1978) and thus leads to decline in virulence.

CHAPTER 4

The effect of successive *in vitro* sub-culturing on pathogenicity of the entomopathogenic fungus, *Metarhizium anisopliae*

4.1 INTRODUCTION

Central to the production of a successful and commercially useful biocontrol agent is the ability of the organism to maintain virulence during inoculum production. Virulence of many fungal strains has been shown to decrease if maintained on artificial media by repetitive sub-culturing (Fox & Jacques, 1958; Kawakami, 1960; Schaerffernberg, 1964). Nagaich (1973) noted that an isolate of *Verticillium lecanii*, pathogenic to aphids, lost its virulence after the second or third sub-culturing on artificial media. Lord & Roberts (1986) found that two isolates of *Lagenidium giganteum* progressively lost the ability to form oospores and zoospores and to infect *Aedes aegypti* larvae, after prolonged culture on sterol-free PYG (peptone, yeast and glucose) medium. Reductions reached statistically significant levels by 15 weeks. However, 15 serial passages through *A. aegypti* restored infectivity.

Several other workers have also reported restored infectivity of attenuated entomopathogenic strains such as *B. bassiana* (Kawakami, 1960; Schaerffenberg, 1964; Aizawa, 1971; Fargues, 1972), *M. anisopliae* (Latch, 1976; Fargues & Robert, 1983), *Entomophaga maimaiga* (Hajek, *et al.*, 1990), *Erynia neoaphidis* (Wilding, 1992) and *Nomuraea rileyi* (Morrow *et al.*, 1989) by passaging the fungi through insect hosts. In contrast, some pathogens such as *M. anisopliae* (Latch, 1976), *Culicinomyces clavosporus* (Sweeny, 1981), *Verticillium lecanii* (Hall, 1980), *Nomuraea rileyi* (Ignoffo *et al.*, 1982) and *Zoophthora radicans* (Dumas & Papierok, 1989) retained their virulence even after prolonged culture *in vitro*. Hartmann & Wasti (1974) reported *Conidiobolus coronatus* to cause little infection of the gypsy moth *Porthetria dispar* after prolonged culturing on artificial media, however, after

passaging the fungus through *Galleria mellonella* three times in serial succession, virulence towards *P. dispar* increased. Similarly, passaging twice in serial succession through *G. mellonella* (Wasti & Hartmann, 1975), increased the virulence of *B. bassiana* towards *G. mellonella.* Kawakami (1960) has also found that the original level of virulence of attenuated entomopathogens such as *B. bassiana*, *Spicaria pracina*, *Oospora destractor, Aspergillus oryzae* and *A. flavus* could be restored after passaging through silkworm larva. It was concluded that the muscardines were rather unstable and could be changed through successive culture on artificial media or through the infection of the silkworm body (Kawakami, 1960).

Although attenuated virulence can be restored through an appropriate host insect (Fargues & Roberts, 1983; Morrow *et al.*, 1989; Prenerova, 1994), it is also possible that the phenomenon of attenuation is irreversible. For example, passaging an attenuated isolate of *V. lenacii* through *Macrosiphoniella sanborni* failed to increase the virulence of the pathogen (Hall, 1980).

Successful pathogenesis is influenced by the biochemical and physiological characteristics of the pathogen during infection. However, it is unlikely that there is a single trait or component of the invasion process that can determine virulence (Butt, 1990). Nevertheless, highly virulent isolates encompass two key attributes: the ability to kill in a relatively short period of time and the ability to cause high mortality at relatively low doses. Pathogenic fungi also produce enzymes such as lipases, chitinases and endoproteases, which are considered to be integral to their pathogenesis. Pr1 is a protease secreted by many hyphomycete fungi including *M. anisopliae* (St Leger *et al.,* 1987a,b) and shown to be a key enzyme in the infection process (St Leger *et al.,* 1988). According to Charnley (1992), if virulence among

fungal isolates were at least partly correlated with cuticle-degrading enzymes, then the primary considerations for strain selection or strain improvement could be recognised. Unfortunately, this link has still to be proved.

Comparative studies of virulent and attenuated forms of a strain or strains could help in the identification of virulence determinants. The results of Chapter 3 have shown that repeated sub-culturing on nutritionally rich media significantly affected the virulence of *M. anisopliae* isolates tested. These findings have negative implications for possible field use of the fungus for biological control of aphids, since any industrial-scale manufacturing method of fungal biocontrol agents is still greatly dependent on *in vitro* production.

The main aims of this study were (i) to determine the effects of serial *in vitro* sub-culturing of three isolates of *M. anisopliae* on SDA and MM media on dose- and time-mortality relationships, (ii) to establish the link between lethal concentration (LC_{50}) and lethal time (LT_{50}) values and virulence of attenuated versus non-attenuated cultures and (iii) to determine the production of the enzyme Pr1 by virulent and attenuated forms of *M. anisopliae*.

4.2 MATERIALS AND METHODS

4.2.1 Virulence tests

Adults of *Myzus persicae*, known to be pathogen-free, were obtained from colonies reared and maintained at Harper Adams University College. For each *M. anisopliae* isolate grown on SDA and MM, conidia were harvested from sub-cultures 1, 3, 5, 7, 9

and 11 and used to prepare a conidial suspension (10^8 conidia ml^1 for inoculum grown on SDA and 10^7 conidia ml^{-1} for inoculum grown on MM) using the procedure described in Chapter 3 (Section 3.2.1). It was this suspension that provided stock inoculum for the dose- and time-mortality studies of virulent and attenuated sub-cultures of *M. anisopliae*.

4.2.1.1 Cumulative mortalities, LC_{50} and LT_{50} studies

Dose-mortality studies were conducted in order to determine the minimal dose at which attenuation (sub-cultures with highest LC_{50} values and lowest cumulative mortality) could initially be detected. To this end, stock conidial suspensions of each isolate and for the respective sub-cultures tested, were serially diluted to provide conidial concentrations of $1.0x10^8$ to $1.0x10^4$ and from $1.0x10^7$ to $1.0x10^3$ conidia ml^{-1} of aqueous 0.03% Tween 80 solution, for both SDA and MM, respectively. The inoculation procedures used were identical to those described in Chapter 2 (Section 2.2.4). Controls consisted of aphids treated with aqueous 0.03% Tween 80 only. The number of dead aphids was recorded daily over a period of 7 days from which LT_{50} values were extrapolated. Mortality of aphids was confirmed to be due to *M. anisopliae* by examining the conidia produced on sporulating cadaver.

4.2.2 Isozyme analysis

Total protein was extracted from 15 mg of freeze-dried conidia isolated from profusely sporulating two-week-old sub-cultures of three isolates of *M.anisopliae* as described in Chapter 2 (Sections 2.2.7 and 2.2.8). Proteins were electrophoresed on a non-denaturing

(IEF) and on a denaturing (SDS-PAGE) 12% polyacrylamide gels. After electrophoresis, slab gels were stained with Coomassie stain.

4.2.3 Enzyme studies

4.2.3.1 Effect of successive sub-culturing on protease (Pr1) production

For each isolate grown on SDA medium, conidia were harvested from 14-day old cultures for each of the sub-cultures 1, 3, 5, 7, 9 and 11 and used to prepare a conidial suspension as described previously. The conidial suspensions (final concentration 10^6 conidia ml medium $^{-1}$) were incubated in a Gallenkamp Orbital Shaker (120 r.p.m.) for 63 hr at 23° C, either in 50 ml Minimal Liquid Medium (MLM: 0.03% K_2HPO_4, 0.03% $MgSO_4.7H_2O$, 0.015% NaCl, 0.03% $CaCl_2.6H_2O$ (saturated), 0.0008% $MnSO_4.4H_2O$, 0.0002% $CuSO_4$ $5H_2O$ and 0.002% $FeSO_4.7H_2O$) supplemented with insect homogenate (cockroaches, *Blaberus discoidalis*) at 1% (w/v), or in 150 ml Sabouraud Dextrose Broth (SDB, Oxoid). After 18 hr incubation, 1 ml samples were taken from triplicate cultures and assessed for germination according to the procedure outlined in Chapter 2 (Section 2.2.3). Sixty-three hr post-inoculation, chymoelastease activity (Pr1) in culture filtrates, which were harvested by vacuum filtration (Whatman no. 1 filter paper), was determined using Suc-$(Ala)_2$-Pro-Phe-pNA as a substrate (St Leger *et al.*, 1988). Twenty µl of culture filtrates were added to 80 µl of 0.1M Tris-HCl (pH 7.9) and the reaction, which was started by adding 100 µl 2 mM substrate in 0.1M Tris-HCl (pH 7.9) was observed as an increase in absorbance at 410 nm at 37 °C at 12 sec intervals over a period of 3 min. One unit of Pr1 activity was equal to 1 µmol of substrate released min^{-1} and was measured using a Multiskan Ascent V1.23 Spectrophotometer.

Final fungal biomass was determined by washing the mycelia with 50 ml distilled water through filter paper (Whatman No 5) and then drying to constant weight at 60 °C.

Protein concentrations of samples were determined by the method of Bradford (1976) using bovine serum albumin (Bio-Rad) as a standard.

4.3.2.2 DNA extraction

Genomic DNA was purified from mycelium produced as described in the previous section. Conidial suspensions were prepared using conidia from sporulating colonies of sub-cultures 1, 3, 5, 7, 9 and 11 grown on SDA. 150 ml Erlenmeyer flasks, containing 50 ml SDB, were inoculated with 1ml of 10^7 conidia ml^{-1} and incubated at 24 °C for 63 hr whilst shaken at 120 r.p.m. Mycelium was then collected aseptically on Whatman No 1 filter paper under vacuum filtration. After washing three times with 50 ml sterilised DW, the mycelium was then dried overnight before being homogenised into a fine powder under liquid nitrogen. A sample (200 mg) of the powder was transferred to a 1.5 ml tube containing 450 µl TES (1.4M NaCl; 100nM Tris-HCl (pH 8.0); 20mM EDTA; 1% SDS), 50 µl 20% CTAB (cetyltrimethylammoniumbromide) and 1 µl β-mercaptoethanol and incubated at 65 °C for 1 hr, agitating every 10 min. The mixture was centrifuged at 5000 r.p.m. for 5 min and the supernatant extracted twice with 500 µl chloroform:isomyl alcohol (24:1). The DNA-containing aqueous phase was collected and mixed with 65 µl cold 3M sodium acetate and 700 µl isopropanol with subsequent incubation for 30 min on ice. The nucleic acids were centrifuged and washed twice with 500 µl ice-cold 70 % ethanol, each wash lasting 40 min. The resultant pellet was air-

dried overnight and then dissolved in 50 µl TE (10mM Tris-HCl (pH 8.0); 1mM EDTA). RNA was digested by the addition of 25 ng Rnase (Sigma, UK) per 100 µl DNA solution and incubated at 37 °C for at least 1 hr. DNA integrity and concentration of the samples was determined by comparison with markers of known concentration by electrophoresis on 0.8% agarose gel.

4.2.2.3 Amplification of Pr1 gene

A portion of the Pr1 gene was amplified using primer pairs (METPR1, METPR2, METPR4, METPR5) and TC-100™ Programmable Thermal Controller (MJ Research, Inc.) with protocols outlined by Leal *et al.* (1997).

The procedure of nested PCR consisted of two successive PCRs. The first PCR was performed using 20 ng of DNA extracted from the sample as template and the outer pair of primers METPR1 (5′ CAC TCT TCT CCC AGC CGT TC 3′) and METPR4 (5′ GTA GCT CAA CTT CTC CAC TC 3′). The second PCR consisted of an aliquot of the first PCR product as template and the inner pair of primers METPR2 (5′ AGG TAG GCA GCC AGA CCG GC 3′) and METPR5 (5′ TGC CAC TAT TGG CCG GCG CG 3′). PCR reactions were carried out in a 96-well microplate (ABgene, Surrey, UK) under controlled conditions: initial denaturation, 4 min at 94 °C, 37 cycles including: denaturation, 1min 94 °C; annealing, 1 min 58 °C; extension 2 min at 72 °C and final extension, 6 min at 72 °C. Each well of the microplate contained reaction volume of 25 µl consisting of 10mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5mM $MgCl_2$, 0.1% Triton X-100, 50 ng of each primer, 200µM of each dNTP (dATP, dCTP, dTTP and dGTP), 0.4 units of DNA Red Hot Polymerase (ROCHE) and 1µl of DNA template. A

second round of nested PCR was performed using the same conditions with the exception for the template where 1µl of the first reaction was used as template. Reaction mixtures were overlaid with a drop of mineral oil (Sigma, UK) and placed in the preheated (60 °C) thermocycler. Reaction products were analysed by electrophoresis in 1.5% agarose gel and ethidium bromide in 0.5 x TBE (45mM Tris base; 45mM boric acid; 1mM EDTA (pH 8.0)) buffer. Nucleic acids were visualised using Molecular Analyst Microsoftware (BioRad, UK).

4.3 RESULTS

4.3.1 Effect of successive sub-culturing on dose-time-mortality relationship and mycosis development of three isolates of *M. anisopliae*

Regression analysis of the data presented in Figure 4.1 showed that mortality was dose-related for all sub-cultures of isolate V245 (Figure 4.1a, $\chi^2(df) = 20.45$ (11), $P < 0.001$), V208 (Figure 4.1b, $\chi^2(df) = 33.84$ (11), $P < 0.001$) and V234 (Figure 4.1c, $\chi^2(df) = 21.26$ (11), $P < 0.001$)) maintained on SDA media. Similar significant relationships between total mortality and dosage were also observed for inocula of all three *M. anisopliae* isolates repeatedly sub-cultured on MM (Figure 4.3a-c). Mortality at 10^4 conidia ml^{-1} was lower than 47% for almost all sub-cultures except for sub-culture 1 (60%) and 5 (65%) of isolate V245 and was not higher than 53% for all sub-cultures of V234 maintained on SDA. The same conidial concentration caused little mortality ranging from 20% (sub-culture 7) to 37% (sub-culture 11) in aphid population treated with isolate V208.

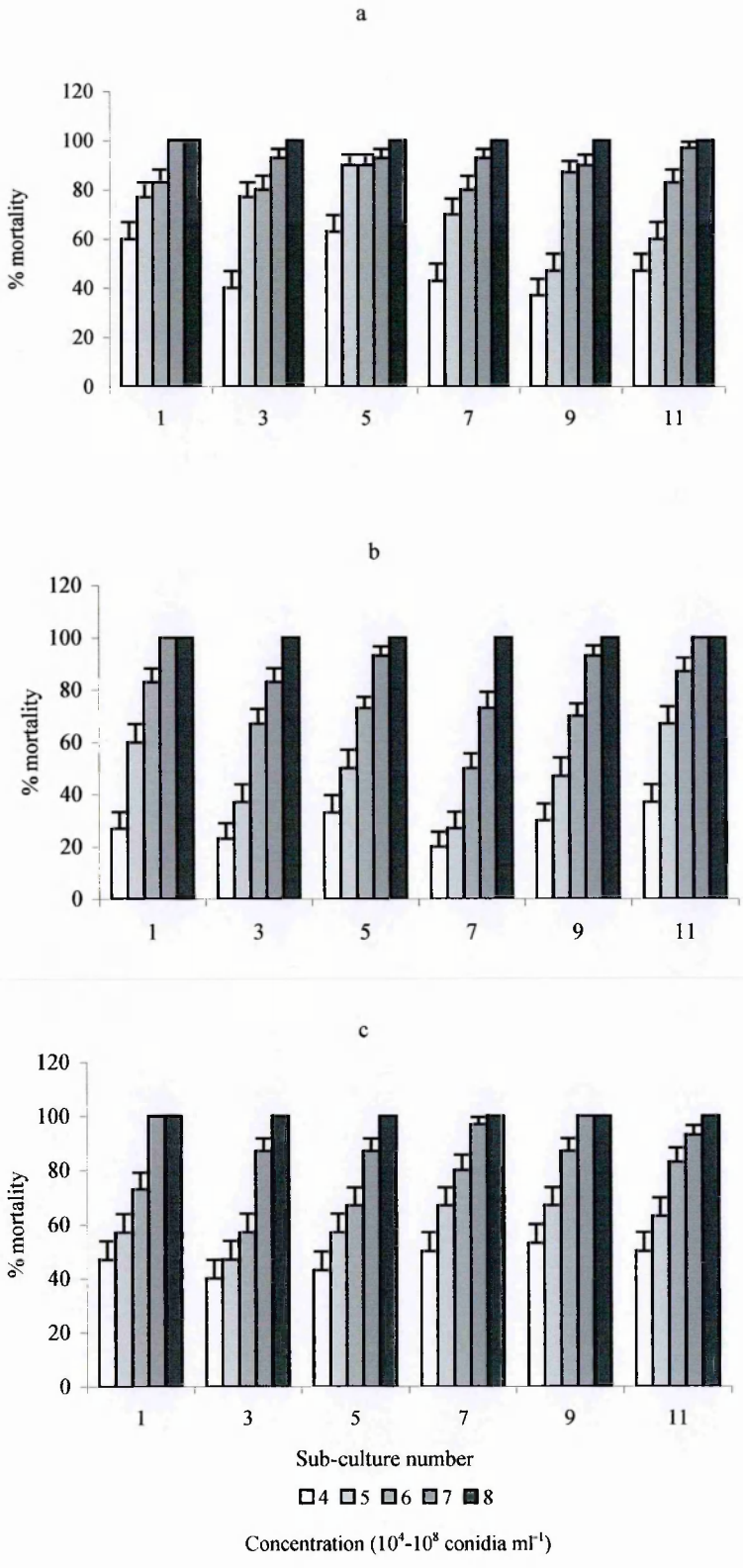


Figure 4.1a-c. Effect of successive *in vitro* sub-culturing on SDA on % mortality of *Myzus persicae* caused by three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) following 7 days incubation at 23 °C at different spore concentrations (10^4-10^8 conidia ml^{-1}).

Although conidia cultured on MM killed more aphids at concentration 10^4 conidia ml^{-1} than conidia from SDA, total mortality for each sub-culture was dependent on the individual response of each isolate to repeated sub-culturing ($F_{10,180}$=7.99, P<0.001; Figure 4.3a-c). For example, % mortalities caused by sub-cultures 7 and 9 of isolate V245 were notably lower then those of sub-cultures 1,3,5 and 11 (Figure 4.2a). At 10^8 conidia ml^{-1} for SDA grown inocula, and at 10^7 conidia ml^{-1} for inocula from MM, mortality was consistently high reaching 100% for all sub-cultures. Control mortality was always less than 6%.

The percentage of dead aphids with external mycoses 14 days after inoculation was significantly lower ($P < 0.01$) than total mortality (Figures 4.2a-c and 4.4a-c). Examination of cadavers indicated that, although aphids exposed to the high and low doses of inoculum differed in their incidence of external sporulation, sporulation appeared to be more prolific on insects exposed to the lower doses. These aphids usually exhibited a dense uniform layer (Plate 4.1a) of conidia compared to the formation of mycelia with irregular sporulation recorded on aphid cuticles exposed to higher inoculum doses (Plate 4.1b).

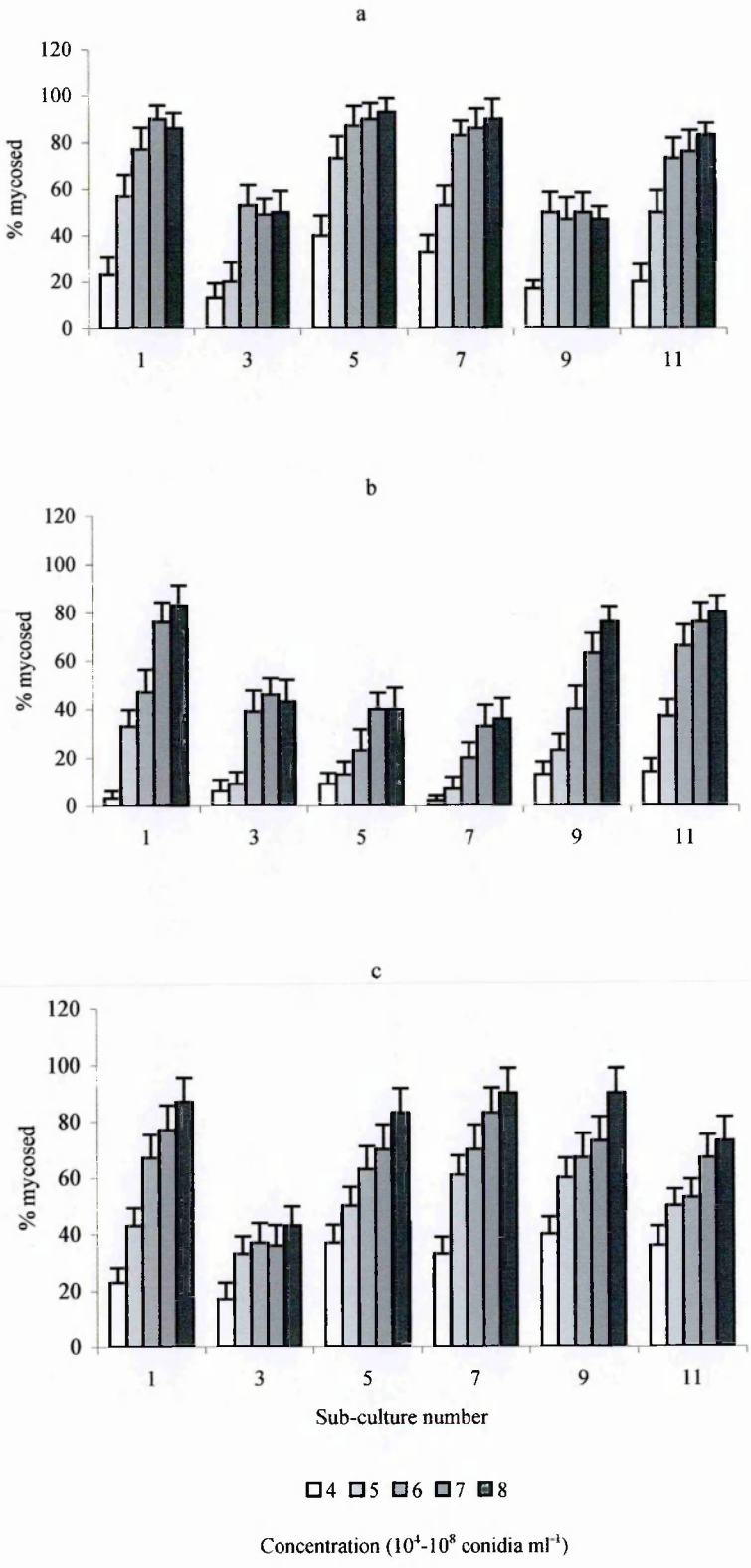


Figure 4.2a-c. Effect of successive *in vitro* sub-culturing on SDA on % mycoses of *Myzus persicae* caused by three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) following 14 days incubation at 23 °C at different spore concentrations (10^4-10^8 conidia ml^{-1}).

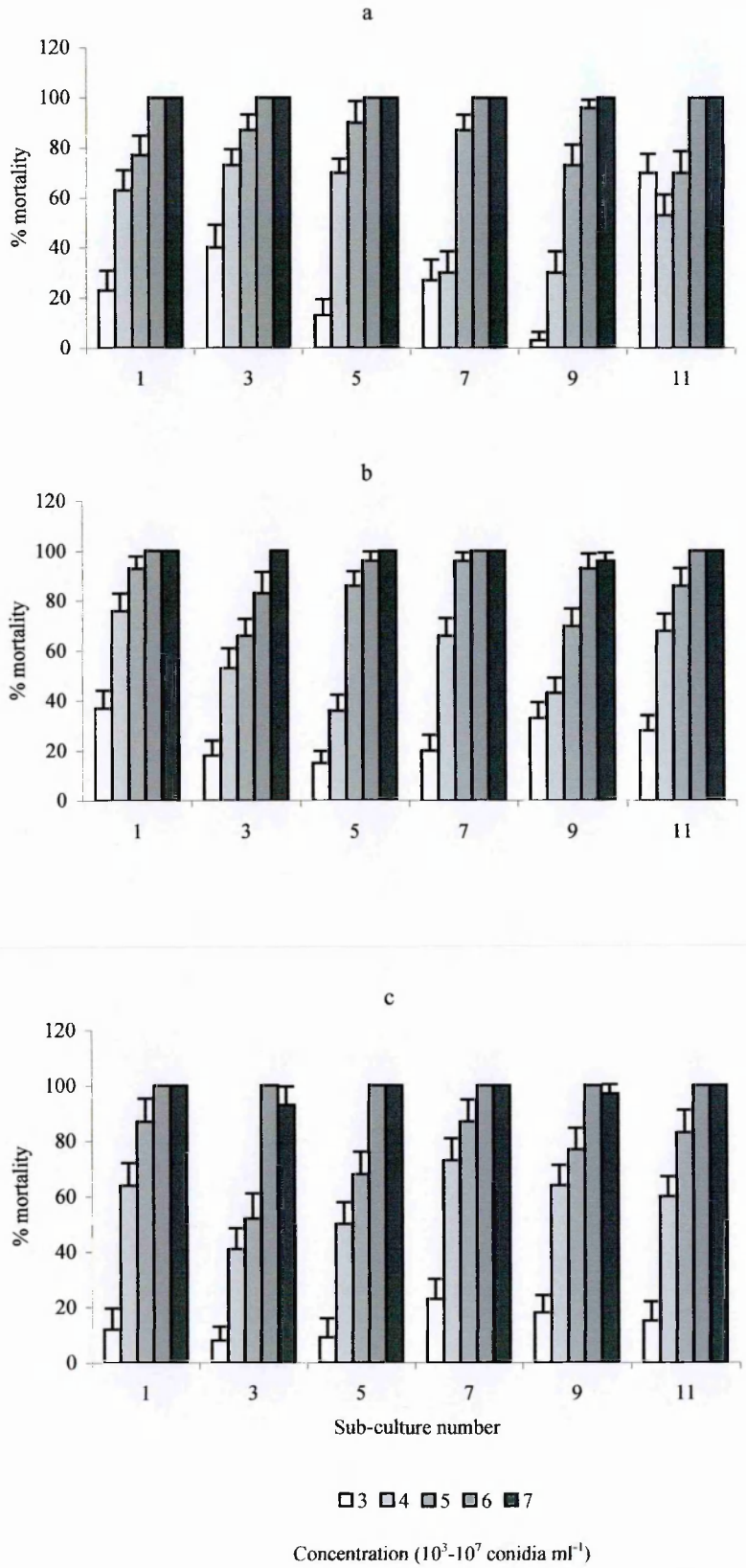


Figure 4.3a-c. Effect of successive *in vitro* sub-culturing on MM on % mortality of *Myzus persicae* caused by three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) following 7 days incubation at 23 °C at different spore concentrations (10^3-10^7conidia ml^{-1})

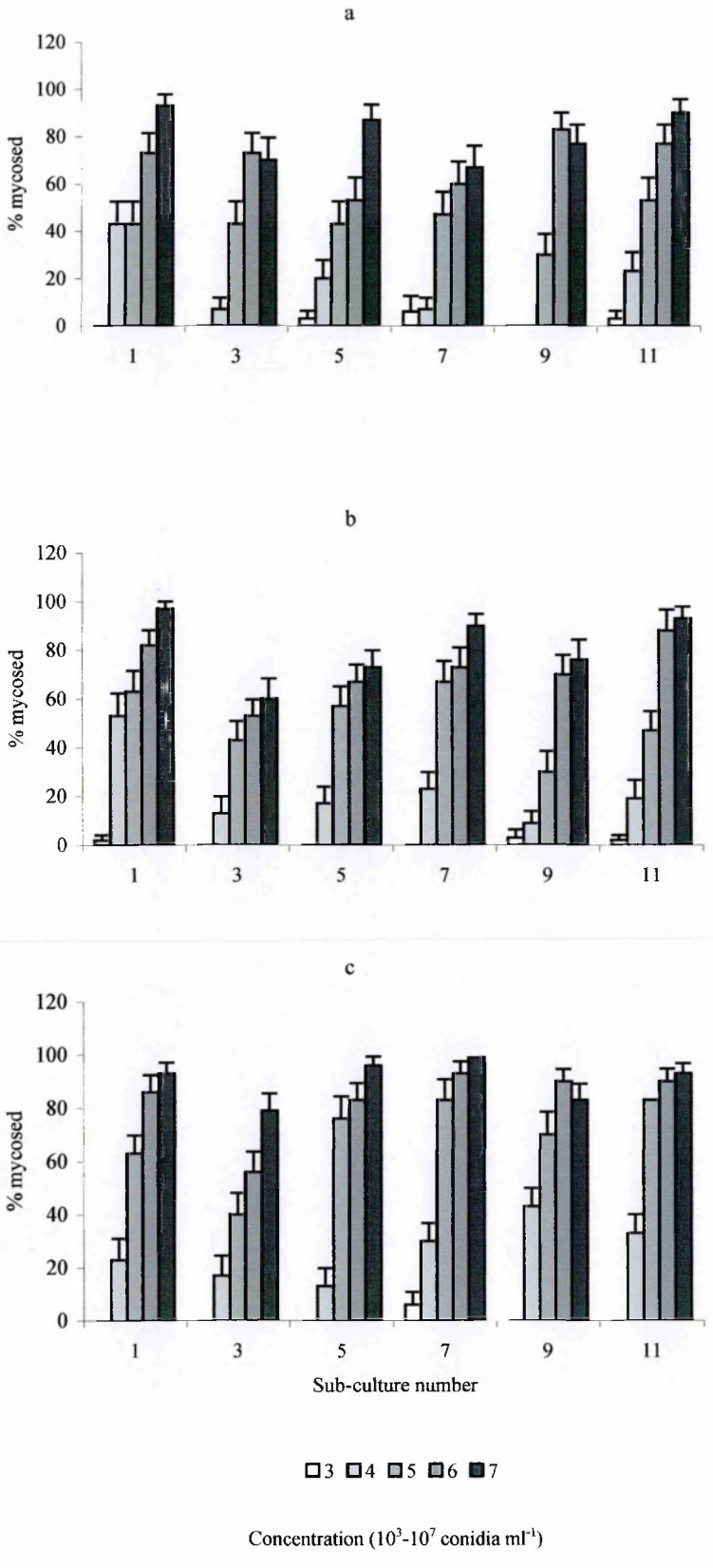


Figure 4.4a-c. Effect of successive *in vitro* sub-culturing on MM on % mycoses of *Myzus persicae* caused by three isolates of *Metarhizium anisopliae* V245 (a), V208 (b) and V234 (c) following 14 days incubation at 23 °C at different spore concentrations (10^3-10^7conidia ml^{-1})

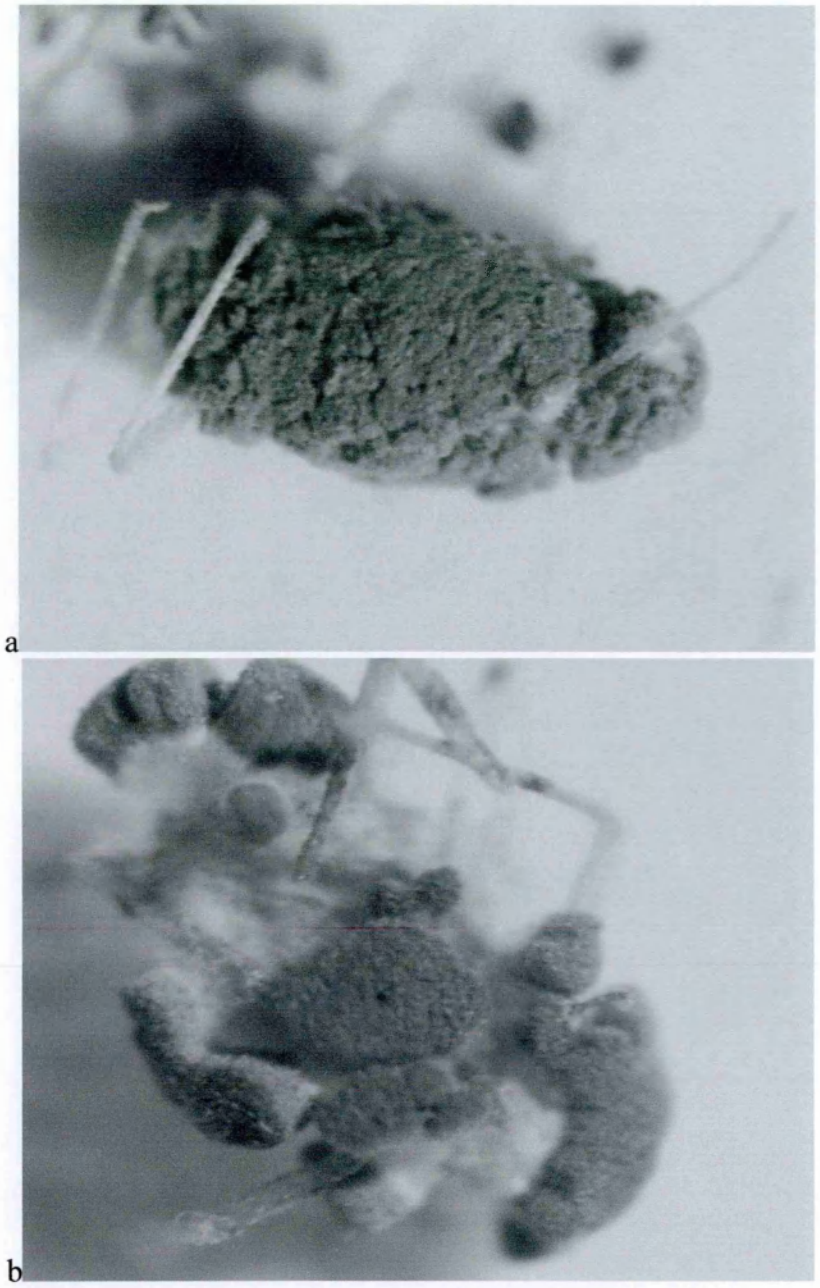


Plate 4.1a-b. External sporulation of V245 isolate on aphid cadavers treated with conidial suspension: **a.** at 10^4 conidia ml^{-1} and **b.** at 10^7 conidia ml^{-1} following 14 days incubation at 23 °C.

Sub-cultures 3 and 9 of isolate V245, sub-cultures 3, 5 and 7 of isolate V208 and sub-cultures 3 and 11 of isolateV234 maintained on SDA (Fig. 4.2a-c) lost the ability to form mycelia/conidia on the exterior of aphid cadavers since a great proportion of dead insects treated with these sub-cultures failed to produce any external mycelia or produced sterile mycelia only. Exposure to higher concentrations of inocula did not revert this lost ability (Fig. 4.2a-c). Although the percentage of mycosis in aphids treated with conidia successively passaged through MM media was generally higher than in aphids treated with conidia passaged through SDA media, continuous sub-culturing of the fungus on MM media significantly affected the ability of sub-cultures 3,7 and 9 of isolate V245, sub-cultures 3,5 and 9 of isolate V208 and sub-cultures 3 and 9 of isolate V234 to cause 100% mycosis (Fig.4.4a-c). For example, only 60% and 67% of aphids treated with sub-cultures 3 and 7 at 10^7 conidia ml^{-1} of isolates V208 and V245, respectively, were positively identified dead due to infection by *M. anisopliae* isolates.

Since the incidence of sporulation on the surface of aphids exposed to low doses was low, conidial production on aphid cadavers was determined for specimens exposed to 10^7 conidia ml^{-1} only. The numbers of conidia produced on dead aphids are presented in Table 4.1. In general, these results reflected the data presented in Figures 4.2a-c and 4.4a-c, where sub-cultures, which showed low level of mycoses, also produced fewer conidia. For example, the number of conidia produced on aphids exposed to sub-culture 11 of isolate V208 from SDA media, was 100 times greater than that produced on aphids exposed to sub-culture 7 of the same isolate. When the fungus was sub-cultured on MM media, the difference in conidial production on aphid cadavers between these two sub-cultures was significantly ($P < 0.001$) reduced by 1.6-fold (Table 4.1).

Table 4.1. Effect of successive *in vitro* sub-culturing on conidia production on dead *Myzus persicae* inoculated with 10^7 conidia ml^{-1} for three isolates of *Metarhizium anisopliae* conidia sub-cultured on SDA and MM media and incubated for 14 days at 23 °C.

Media/ Sub-culture	Isolate		
	V245 Mean number of conidia $aphid^{-1}$ (SE)[a]	V208 Mean number of conidia $aphid^{-1}$ (SE)	V234 Mean number of conidia $aphid^{-1}$ (SE)
SDA			
1	1.9×10^3 (0.8×10^2)	8.9×10^2 (9.3×10)	1.1×10^4 (0.7×10^3)
3	3.3×10^2 (1.8×10^2)	0.7×10^2 (3.4×10)	1.8×10^3 (1.1×10^2)
5	2.3×10^3 (0.6×10^2)	0.6×10^2 (2.5×10)	9.2×10^3 (2.8×10^2)
7	2.1×10^3 (0.9×10^2)	0.1×10^2 (0.6×10)	1.6×10^4 (0.9×10^3)
9	1.7×10^2 (1.1×10^2)	6.6×10^2 (4.3×10)	9.0×10^3 (3.3×10^2)
11	1.3×10^3 (0.3×10^2)	1.0×10^3 (3.2×10^2)	8.4×10^3 (1.9×10^2)
MM			
1	4.8×10^4 (3.5×10^3)	6.8×10^4 (2.9×10^3)	7.4×10^4 (4.1×10^3)
3	3.2×10^4 (3.7×10^3)	1.8×10^4 (5.5×10^3)	6.8×10^4 (3.6×10^3)
5	4.6×10^4 (3.3×10^3)	4.6×10^4 (3.5×10^3)	7.9×10^4 (4.5×10^3)
7	2.8×10^4 (2.5×10^3)	5.9×10^4 (1.3×10^3)	9.2×10^5 (1.5×10^4)
9	3.7×10^4 (2.8×10^3)	3.8×10^4 (2.8×10^3)	5.3×10^4 (1.3×10^3)
11	4.5×10^4 (3.6×10^3)	6.0×10^4 (2.4×10^3)	5.8×10^4 (1.7×10^3)

[a]SE = standard errors of means.

Conidial concentrations ranging between 1.0×10^4 to 1.0×10^8 conidia ml^{-1} for sub-cultures 1, 3, 5, 7, 9 and 11 from SDA media and between 1.0×10^3 to 1.0×10^7 conidia ml^{-1} for sub-cultures 1, 3, 5, 7, 9 and 11 from MM media were used to establish the best dose for subsequent studies and to determine the minimal concentration at which attenuation could be initially detected in all three isolates studied. The LD_{50}/LC_{50} is defined as the dose/concentration of agent (chemical or biological) per insect that will produce death in half the test animals (Maddox, 1982). However, in the case of pathogens, the relationship between mortality and dose is more complicated, and hence, a time element needs to be considered since mortality is a function of both dose and time. Furthermore, since mortality may vary with time (and other factors such as

relative humidity, age of target host, length of the photophase, and temperature), a more meaningful approach was to estimate the time it would take for 50% of the test insects to be killed as a function of dose (hereafter called LT_{50}).

Bioassay results (Tables 4.2 and 4.3) obtained from this study clearly demonstrated that subtle changes in virulence following sub-culturing on either SDA or MM media, were best detected at lower inoculum concentrations (i.e., 10^4 or 10^5 conidia ml^{-1}). Estimated LC_{50} values for inocula sub-cultured on SDA ranged from 0.49 x 10^4 conidia ml^{-1} (sub-culture 5) to 6.5 x 10^4 conidia ml^{-1} (sub-culture 9) for isolate V245, from 0.62 x 10^5 conidia ml^{-1} (sub-culture 1) to 7.9 x 10^5 conidia ml^{-1} (sub-culture 7) for isolate V208 and from 0.97 x 10^4 conidia ml^{-1} (sub-culture 9) to 9.7 x 10^4 conidia ml^{-1} (sub-culture 3) for isolate V234 (Table 4.2).

Table 4.2. Effect of successive sub-culturing on median lethal concentration (LC_{50}) obtained for three isolates of *Metarhizium anisopliae* sub-cultured on SDA and tested against *Myzus persicae* following 7 days incubation at 23 °C.

Sub-culture	Isolate		
	V245 LC_{50}[a] (conidia ml^{-1} at 10^4 ± 95% confidence intervals)	V208 LC_{50} (conidia ml^{-1} at 10^5 ± 95% confidence intervals)	V234 LC_{50} (conidia ml^{-1} at 10^4 ± 95% confidence intervals)
1	0.75 ±0.26	0.62 ± 0.20	5.04 ± 0.25
3	2.48 ±0.23	3.35 ± 0.17	9.72 ± 0.25
5	0.49 ±0.28	0.92 ± 0.18	6.26 ± 0.29
7	2.95 ±0.23	7.89 ± 0.17	2.33 ± 0.30
9	6.50 ±0.22	0.99 ± 0.18	0.97 ± 0.29
11	2.77 ±0.24	3.62 ± 0.20	1.87 ± 0.32

A dramatic decline in virulence (as measured by LC_{50} values) ($P < 0.001$) for inocula sub-cultured on MM media was observed for sub-cultures 7 and 9 for both V245 (4.1 x 10^4 and 89.7 x 10^4 conidia ml^{-1}, respectively) and V234 (2.5 x 10^4 and 3.4 x 10^4 conidia

ml^{-1}, respectively) isolates and for sub-culture 9 (8.3×10^4 conidia ml^{-1}) of isolate V208 (Table 4.3).

Table 4.3. Effect of successive sub-culturing on median lethal concentration (LC_{50}) obtained for three isolates of *Metarhizium anisopliae* sub-cultured on MM and tested against *Myzus persicae* following 7 days incubation at 23 °C.

Sub-culture	Isolate		
	V245 LC_{50}[a] (conidia ml^{-1}at 10^4 ± 95% confidence intervals)	V208 LC_{50} (conidia ml^{-1}at 10^4 ± 95% confidence intervals)	V234 LC_{50} (conidia ml^{-1}at 10^4 ± 95% confidence intervals)
1	0.77 ± 0.17	0.82 ± 0.33	0.32 ± 0.19
3	0.26 ± 0.28	0.96 ± 0.20	0.84 ± 0.23
5	0.66 ± 0.18	0.67 ± 0.18	0.66 ± 0.20
7	4.08 ± 0.15	0.69 ± 0.43	2.48 ± 0.44
9	89.7 ± 0.14	8.31 ± 0.83	3.37 ± 0.63
11	0.90 ± 0.16	0.97 ± 0.21	0.62 ± 0.17

Conversely, the most significant ($P < 0.001$) discrepancies between LT_{50} values of all sub-cultures were observed at higher inoculum concentrations (10^7 conidia ml^{-1}) for all three isolates tested, when sub-cultured on SDA media (Fig. 4.5a-b). Initial death of aphids was observed on day 2 for non-attenuated sub-cultures 1 and 5 of isolate V245 and sub-culture 1 of isolate V208, and on day 3 for sub-culture 9 of isolate V234 resulting in mean LT_{50} value of approximately 3.7 ± 0.9 days. For the most attenuated sub-cultures 3 and 9 (LT_{50} = 4.8 ± 0.9 days for both) of V245 and sub-cultures 3 (LT_{50} = 5.6 ± 1.0 days) and 5 (LT_{50} = 5.2 ± 1.3 days) of V234, the initial death was observed on day 3. For sub-cultures 3 (LT_{50} = 5.4 ± 1.1 days) and 7 (LT_{50} = 6.1 ± 1.0 days) of V208, initial death was observed on day 3 and 4, respectively. However, if *M. anisopliae* isolates were sub-cultured on MM media, then statistical differences between LT_{50} values of all sub-cultures tested were most pronounced at 10^3 conidia ml^{-1}

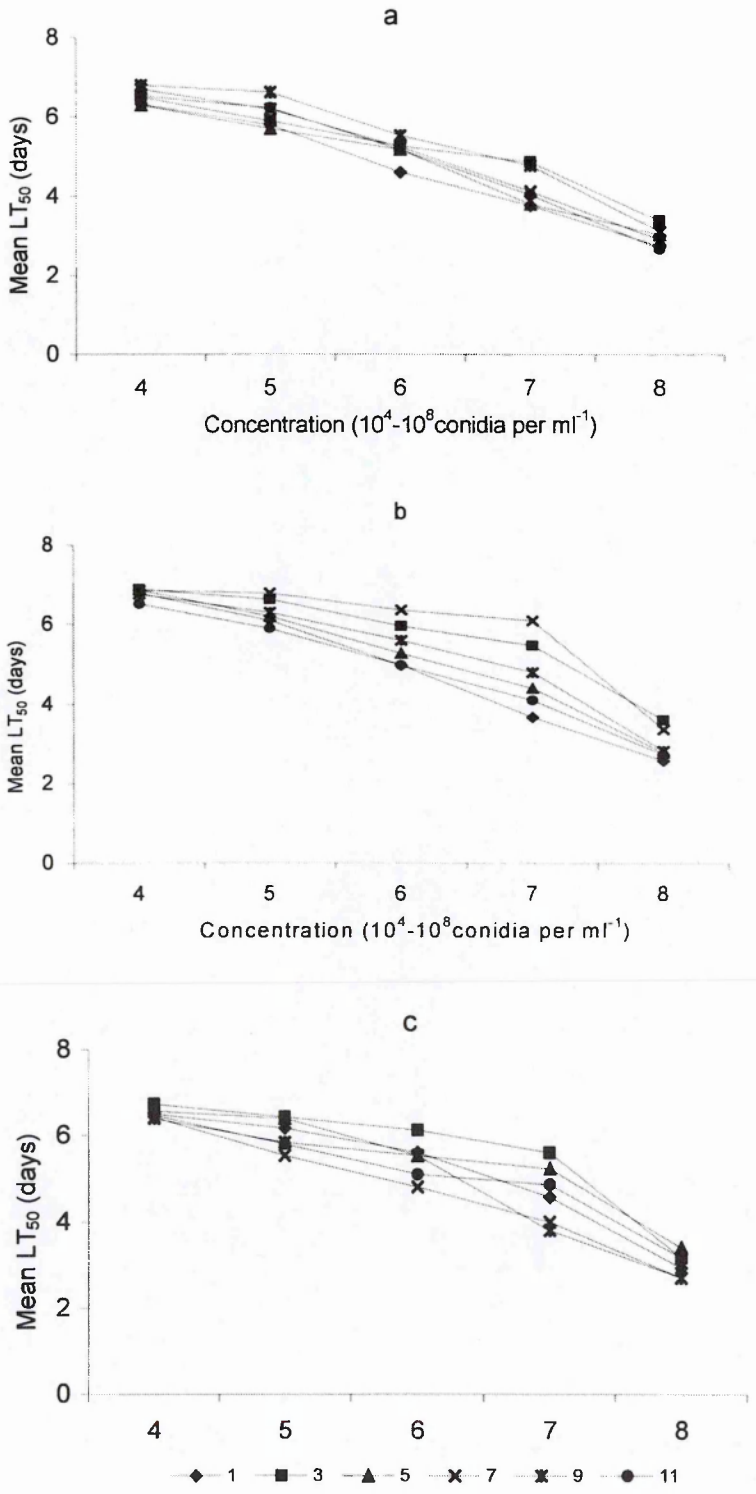


Figure 4.5a-c. Effect of successive *in vitro* sub-culturing on SDA on LT_{50} values obtained for three isolates of *Metarhizium anisopliae* V245 (a) ($p<0.01$ at 10^6-10^8 conidia ml^{-1}), V208 (b) ($p<0.01$ at 10^5-10^7 conidia ml^{-1}) and V234 (c) ($p<0.01$ at 10^5-10^8 conidia ml^{-1}) at different spore concentrations (10^4-10^8 conidia ml^{-1}) following 7 days incubation at 23 °C.

for isolate V245, at 10^4 conidia ml^{-1} for isolate V208 and at 10^5 conidia ml^{-1} for V234 isolate (Fig. 4.6a-c). For example, the lowest LT_{50} value of 5.8 days was obtained for sub-culture 3 of V245 isolate and the highest was recorded for sub-culture 9 with an LT_{50} value of 11.3 days at 10^3 conidia ml^{-1} (Fig.4.6a). A 10000-fold increase in dose resulted in a significant decrease in LT_{50} falling to only 2.4 days for sub-culture 3 and 2.9 days for sub-culture 9. Conidia of sub-culture 11 of isolate V208 (Fig.4.6b) took 4.4 days to kill 50% aphids investigated, whereas conidia of sub-culture 3 needed 6.8 days to infect and kill the same number of insects when treated with 10^4 conidia ml^{-1}. Likewise, conidia of sub-culture 3 required 2.3 days more than sub-culture 1 of V234 isolate to cause death in half the test individuals at 10^5 conidia ml^{-1} (Fig.4.6c).

4.3.2. Electrophoretic studies

Protein analysis of IEF gels indicated not only distinct banding differences between isolates (Plate 4.2a-b) but also reflected quantitative differences (as measured by intensity of individual bands) between sub-cultures of respective isolates. The intensity of protein bands for isolate V208 (Plate 4.2b) was shown to be higher than it was for isolates V234 (Plate 4.2c) or V245 (Plate 4.2a) suggesting that V208 contained more water-extractable proteins than the other two isolates. The intensity of protein bands of isolate V208 remained unchanged during repeated sub-culturing on SDA medium. Moderately strong bands of V245 proteins, however, became weaker with each successive sub-culture, whereas intensity of V234 bands became stronger for sub-cultures 7, 8 and 9, suggesting that the three isolates differ in their susceptibility to sub-culturing on artificial media.

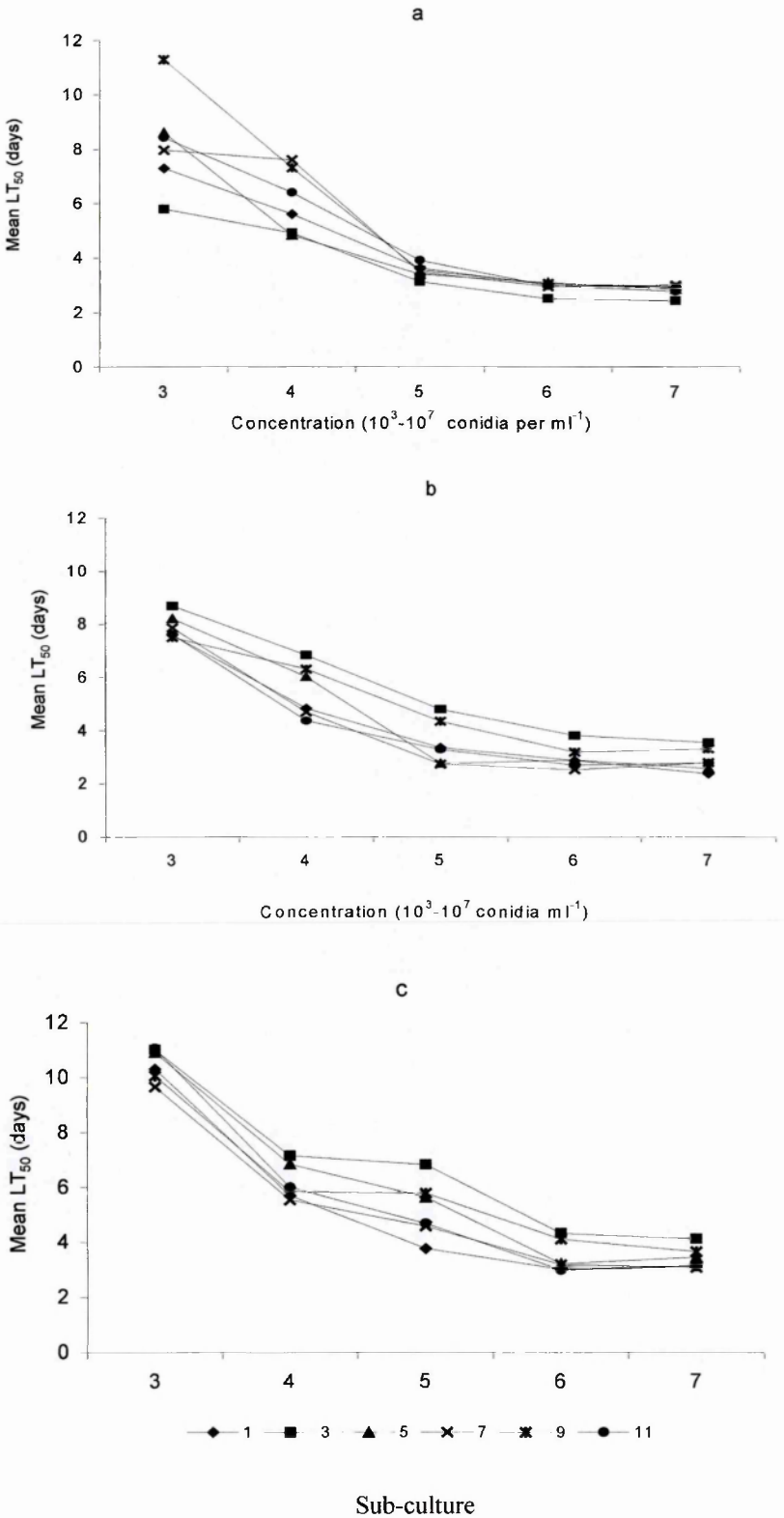


Figure 4.6a-c. Effect of successive *in vitro* sub-culturing on MM on LT_{50} values obtained for three isolates of *Metarhizium anisopliae* V245 (a) ($p<0.01$ at 10^3-10^4 conidia ml^{-1}), V208 (b) ($p<0.01$ at 10^4-10^6 conidia ml^{-1}) and V234 (c) ($p<0.05$ at

4.3.3 Enzymatic analysis

4.3.3.1 Pr1 production: quantitative enzyme assay

Minimal liquid medium (MLM) containing cockroach homogenate as the sole carbon and nitrogen source allowed 100% conidial germination to occur in all sub-cultures of all three isolates investigated after 18-hour incubation at 23° C (Table 4.4). This medium also induced Pr1 production, yielding high concentrations of the enzyme within 63 hr of incubation (Table 4.5). The amounts of Pr1 produced were not significantly different ($F_{2,34} = 1.7$, $P>0.05$) between isolates and the induction response of each isolate to Pr1 production was not affected ($F_{5,34} = 1.45$, $P>0.05$) by successive sub-culturing. However, the amount of Pr1 produced by an individual sub-culture was shown to depend on a significant link ($F_{10,34} = 2.64$, $P<0.05$) between the fungal isolate and its response to sub-culturing. For example, the highest chymoelastease (Pr1) activity (2.56 units) was observed in culture filtrate of sub-culture 11for isolate V234 whereas this activity was shown to be the lowest (0.43 units) in the same sub-culture filtrate for isolate V208.

Protein assays also revealed that the concentration of proteins (Table 4.5) released into culture media was similar for all sub-cultures ($F_{5,34} = 2.30$, $P>0.05$) of all three isolates tested ($F_{2,34} = 1.93$, $P>0.05$) and significantly dependent on the interaction between fungal organisms and their response to successive sub-culturing ($F_{10,34} = 3.26$, $P<0.01$). The biomass of all successive sub-cultures for all isolates grown in this media was also comparable ($P>0.05$) (Table 4.5) suggesting similar rates of growth.

In comparison with basal medium containing cockroach homogenate (MLM), SDB had completely repressed the production of Pr1. However, the speed of germination of individual sub-cultures in this medium was influenced by the ability of each isolate to withstand continued sub-culturing (Table 4.4). For example, sub-cultures 3 and 7 of V208 were the slowest of all to germinate, resulting in 61% and 69% germination respectively. Similarly, 78% and 86% was recorded, respectively, for sub-cultures 3 and 9 of V245, and 91% and 89% germination occurred, respectively, in sub-cultures 3 and 5, of isolate V234.

Table 4.4. Percentage germination of three isolates of *Metarhizium anisopliae* conidia produced by repeated sub-culturing on SDA following 18 h incubation period in MLM amended with cockroach homogenate and SDB at 23 °C.

Isolate	Sub-culture	Percentage germination (±SE)	
		MLM[a]	SDB[b]
V245	1	100 (0.0)	99.8 (0.8)
	3	100 (0.0)	78.2 (1.2)
	5	100 (0.0)	96.3 (0.6)
	7	100 (0.0)	100 (0.0)
	9	100 (0.0)	86.4 (1.0)
	11	100 (0.0)	91.0 (1.1)
V208	1	100 (0.0)	87.8 (2.2)
	3	100 (0.0)	61.4 (3.3)
	5	100 (0.0)	93.2 (1.6)
	7	100 (0.0)	68.7 (0.9)
	9	100 (0.0)	90.2 (0.5)
	11	100 (0.0)	83.2 (2.3)
V234	1	100 (0.0)	98.8 (0.3)
	3	100 (0.0)	90.8 (3.2)
	5	100 (0.0)	89.3 (3.7)
	7	100 (0.0)	96.7 (1.1)
	9	100 (0.0)	97.7 (0.8)
	11	100 (0.0)	93.5 (0.5)

[a] MLM = Minimal Liquid Medium amended with 10g l^{-1} of cockroach homogenate.
[b] SDB = Sabouraud Dextrose Broth.

Table 4.5. Effect of *in vitro* sub-culturing on SDA on Prl activity, total protein and biomass of three isolates of *Metarhizium anisopliae* cultured in MM liquid media containing cockroach homogenate for 63 hr at 23 °C.

Isolate	Sub-culture	Prl (μmol pNA min^{-1} ml^{-1})[a]	Protein (mg ml^{-1})	Biomass (g ml^{-1})
V245	1	-5.03 (1.21)	0.12	0.0045
	3	-3.80 (2.31)	0.14	0.0063
	5	-5.13 (1.2)	0.14	0.0058
	7	-4.35 (1.71)	0.10	0.0059
	9	-4.59 (1.14)	0.09	0.0052
	11	-3.71 (2.42)	0.11	0.0055
V208	1	-4.20 (1.54)	0.15	0.0062
	3	-4.89 (0.76)	0.16	0.0061
	5	-4.86 (0.92)	0.12	0.0061
	7	-4.81 (0.96)	0.12	0.0052
	9	-4.89 (0.78)	0.50	0.0054
	11	-5.45 (0.43)	0.11	0.0056
V234	1	-5.32 (0.56)	0.11	0.0044
	3	-5.99 (0.31)	0.14	0.0047
	5	-5.13 (0.84)	0.11	0.0054
	7	-4.04 (1.88)	0.08	0.0039
	9	-4.76 (0.86)	0.18	0.0063
	11	-3.66 (2.56)	0.21	0.0041
LSD	Isol	0.5; $p>0.05$	0.30; $p>0.05$	0.0009; $p>0.05$
	Subc	0.7; $p>0.05$	0.02; $p>0.05$	0.001; $p>0.05$
	Isol*Subc	1.2; $p<0.05$	0.03; $p<0.01$	0.002; $p>0.05$

[a]-activities are expressed as μmol of nitroaniline released ml^{-1} min^{-1} from suc-$(Ala)_2$-Pro-Phe-pNA and are the transformed (complementary log-log) means of three replicate cultures. Numbers in parentheses are untransformed means.

4.3.3.2 *prl* gene expression: nested PCR

The objective of this experiment was to compare *prl* gene expression in non-attenuated and attenuated sub-cultures of the three *M. anisopliae* isolates cultured in SDB. To this end, a PCR technique based on the amplification of a portion of the gene encoding a major protease, the subtilisin Prl, was used. To produce PCR product corresponding

with part of the *pr1* gene, a two-stage reaction (nested PCR) and two sets of primers: METPR1+ METPR4 (flanking the gene) and METPR2+METPR5 (inner region of the gene) were required. As a result of such a reaction, the primers amplified a strong, single 1.2-kb fragment (Plate 4.3) from genomic DNA extracts of all sub-cultures (attenuated and/or non-attenuated) of all three isolates V245, V208 and V234. Although the level of protease (Pr1) activity in SDB medium remained undetectable after 63 hr incubation period, the mycelia collected from such medium clearly showed expression of the *pr1* gene. The expression was independent of sub-culturing (Plate 4.3).

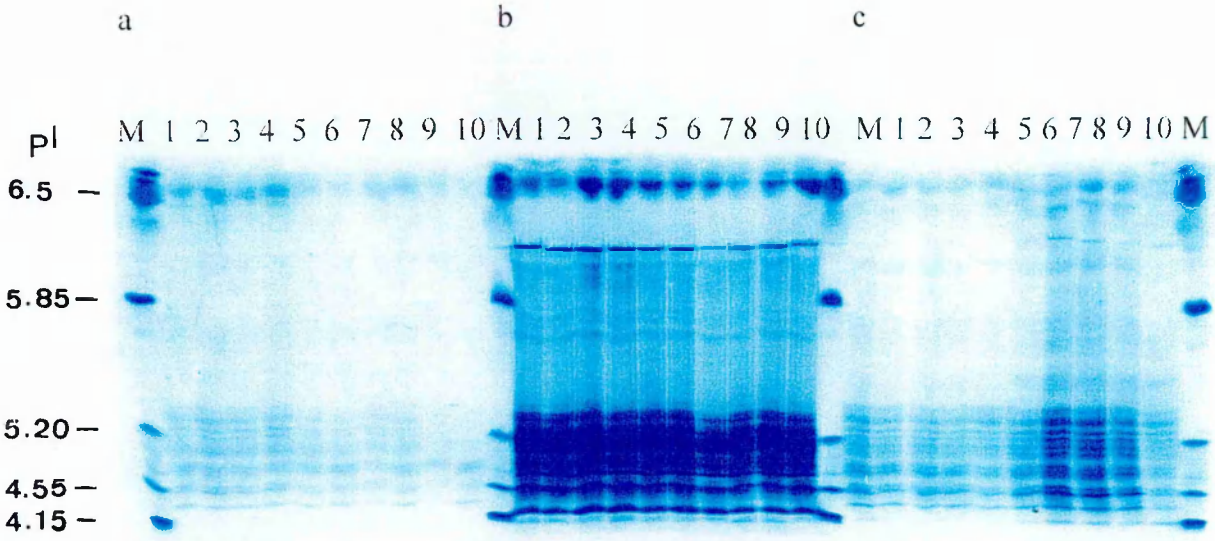


Plate 4.2. Analytical IEF (pH 4-6.5) of water-extracted proteins from conidia of three isolates of *Metarhizium anisopliae:* V245 (a), V208 (b) and V234 (c) harvested from sub-cultures: 1 (lane 1), 2 (lane 2), 3 (lane 3), 4 (lane 4), 5 (lane 5), 6 (lane 6), 7 (lane 7), 8 (lane 8), 9 (lane 9) and 10 (lane 10). M (pI marker proteins).

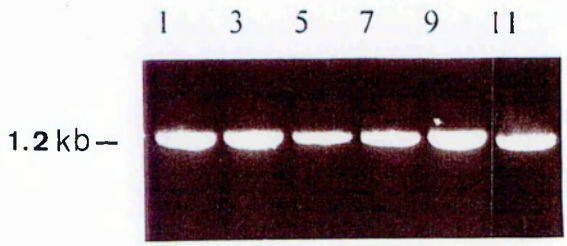


Plate 4.3. Detection of Pr1 PCR product of *Metarhizium anisopliae* isolate V208 in DNA extracts from mycelia grown in Sabouraud Dextrose Broth of sub-cultures: 1 (lane 1), 3 (lane 2), 5 (lane 3), 7 (lane 4), 9 (lane 5) and 11 (lane 6).

4.4 DISCUSSION

This study has shown that *Metarhizium anisopliae* isolates V245, V208 and V234 were pathogenic to *M. persicae* at all concentrations ranging between 10^3 and 10^7 conidia ml^{-1} for inocula produced on Minimal Medium and between 10^4 and 10^8 conidia ml^{-1} for inocula produced on Sabouraud Dextrose Agar. Furthermore, it has also been shown that virulence (as measured by LT_{50} values) increased with increasing dose rate. Exposure to, and infection by, *M. anisopliae* at low inoculum doses had the least impact on total mortality and on the number of dead aphids developing mycosis (Figures 4.1a-c - 4.4a-c). High mortalities (70%-100%) were consistently observed in aphid populations treated with *M. anisopliae* isolates at 10^5-10^7 conidia ml^{-1} for conidia sub-cultured on MM and at 10^6-10^8 conidia ml^{-1} for conidia grown on SDA media. These observations support earlier findings (Butt *et al.*, 1994; Butt *et al.*, 1995, Ibrahim *et al.*, 2002) where isolates of V245 and V208 were shown to cause 100% death of *M. persicae* and *Lipaphis erysimi* within 4-7 days post-inoculation using 10^7 conidia ml^{-1}. However, even under favourable conditions, individual conidia of the common hyphomycete entomopathogens such as *M. anisopliae* and *B. bassiana* during infectivity trials often provide variable LC_{50} values (Wraight & Carruthers, 1999). This may account for the high inoculum doses (10^{13} and 10^{14} conidia ha^{-1}) typically required to control pests under field conditions (Bartlett & Jaronski, 1988).

Due to the large quantities required, inoculum is often prepared and stored for later use during successive bioassays or field trials. However, storing large quantities of

inoculum can only be a provisional measure, since, when stocks have been exhausted, fresh inoculum has to be prepared through repeated sub-culturing on artificial media. Prolonged or repeated *in vitro* sub-culturing is known to cause instability and attenuation (reduction or loss) of virulence in many entomopathogenic fungal species such as *Spicaria pracina, Isaria fumosorosea, Oospora destructor, Aspergillus flavus, Aspergillus oryzae, B. bassiana* (Kawakami, 1960, Aizawa, 1974; Wasti & Hartmann, 1975), *V. lecanii* (Nagaich, 1973), *M. anisoplie* (Fargues & Roberts, 1983; Chapter 3), *N. rileyi* (Morrow *et al.,* 1989), *Entomophaga maimaga* (Hajek *et al.,* 1990), *E. neoaphidis* (Wilding *et al.,* 1992). This attenuation of virulence may necessitate field applications of fungal inocula at even higher dose rates in order to maintain consistent levels of virulence in the field.

An understanding of the phenomenon of attenuation in *M. anisoplia,* therefore, became imperative since it would help to obtain a better understanding of the pathogen traits and would ensure that only stable, virulent isolates were used for mass cultivation (Tillemans *et al.,* 1992). Such knowledge would also provide a more efficient control of agricultural pests, either through manipulation of media compositions, through physiological manipulations (Lane *et al.,* 1991a, 1991b; Jackson and Schisler, 1992; Hallsworth and Magan, 1994a, 1994b, 1994c 1995; Ibrahim *et al.*, 2002) or through manipulation of fungal genes (St Leger *et al.*, 2002).

Since reduction in virulence (as measured by low total mortality and increased LT_{50} values at 10^5 conidia ml^{-1}), resulting from successive sub-culturing on SDA medium was noted for sub-cultures 3, 7 and 9 of isolate V245, sub-cultures 3 and 7 of isolate V208 and for sub-cultures 3 and 5 of V234 isolate (Chapter 3, Section 3.3.4), it was

necessary to employ a wider range of conidial concentrations in order to determine a minimum dose at which attenuation could first be perceived. The data obtained from the present study are in complete agreement with the data from previous studies (Butt *et al.*, 1995) and clearly demonstrate that aphid mortality is dose related (Figures 4.1a-c and 4.3a-c). This relationship does not overtly appear to be affected by sub-culturing or media used.

Frequent sub-culturing on artificial media has been previously shown to affect LC_{50} values (Ignoffo *et al.*, 1982). Although not significant, a 2-fold increase (from 16.2 to 31.9 conidia mm^2) in the LC_{50} of the *in vitro* produced conidia of *Nomuraea rileyi* against *Trichoplusia ni* larvae was observed during the 12^{th} to 18^{th} serial passages on Sabouraud Maltose Agar (Ignoffo *et al.*, 1982). Sub-culture 1 of *M. anisopliae* V245 isolate in this study required 7500 conidia ml^{-1} to kill half of test aphids. This requirement increased significantly by more than 3-fold for sub-culture 3 and more than 8.5-fold for sub-culture 9 when produced on SDA or more than a 100-fold for sub-culture 9 when produced on MM. These data corroborated the results from Chapter 3 that attenuation of *M. anisopliae* isolates associates with changes in adhesion properties of conidia.

Repeated sub-culturing can also affect the time that is taken from initiation to completion of disease development (latent period). For example, bioassays conducted against gypsy moth, *Lymantria dispar*, using *Entomophaga maimaiga* protoplasts demonstrated a significant 2 day increase in disease incubation time after 15 *in vitro* passages (Hajek *et al.*, 1990). Similarly, a total of more than 6 days required for sub-culture 3 of isolate V208 compared to that of 3.8 days of sub-culture 1 to achieve death

in 50% of all aphids exposed to 10^7 conidia ml^{-1} grown on SDA (Fig. 4.5b). Furthermore, aphids exposed to conidia of isolate V234 sub-cultured once on MM media, died 2.3 days earlier than aphids exposed to the same conidia sub-cultured 3 times in succession (Fig.4.6c). Even though attenuation of virulence in *M. anisopliae* isolates, as measured by LT_{50} values, was evident at low doses (Figs 4.5a-c and 4.6a-c), the best doses at which significant discrepancies in LT_{50}'s could be easily detected were 10^7 conidia ml^{-1} for inocula produced on SDA and 10^3-10^5 conidia ml^{-1} for inocular grown on MM media. However, it must be remembered that at 10^7 conidia ml^{-1} and above mortality was consistently high for all sub-cultures of all three isolates, suggesting that attenuation of virulence could be overlooked when high conidial concentrations are applied.

How quickly a pathogen becomes attenuated clearly depends on the isolate. A strain of *Verticillium lecanii* pathogenic to aphids, *Macrosiphoniella sanborni,* lost its virulence after the second or third *in vitro* sub-culturing (Nagaich, 1973). A substantial loss of virulence was also observed for 6 isolates of *Beauveria bassiana* after 2 passages on Bacto Mycological Agar (Samšiňáková, A. & Kálalová, 1983). One of these isolates completely lost its pathogenicity after three successive sub-cultures. Increased LC_{50} and LT_{50} values were observed by sub-culture 3 for all three isolates examined in present study, suggesting that the nature of *M. anisopliae* does change after at least 2 sub-cultures on both media tested. A more gradual decline in virulence was also seen in many other entomopathogenic fungi (Ignoffo *et al.*, 1982; Lord & Roberts, 1986; Morrow *et al.*, 1989; Hajek *et al.*, 1990). Rapid or slow, the reduction in virulence of attenuated strains can be prevented by passaging through an appropriate host, e.g. *B. bassiana* (Kawakami, 1960; Schaerffenberg, 1964; Aizawa, 1971; Fargues, 1972), *M.*

anisopliae (Latch, 1965; Fargues & Robert, 1983), *Entomophaga maimaiga* (Hajek, *et al.*, 1990), *Nomuraea rileyi* (Morrow *et al.*, 1989), *Erynia neoaphidis* (Wilding, 1992). Nevertheless, virulence of attenuated isolates can also be regained without passaging through insects (Chapter 3 and present study). Why recovery of virulence occurs in the present or absence (see Chapter 3 and present data) of passaging through a host insect still remains unknown.

The mechanisms for reduced virulence have seldom been explored among those fungi with decreased virulence after sub-culturing. Ignoffo *et al.* (1982) studied the electrophoretic banding patterns of *Nomuraea rileyi* after 18 *in vitro* passages on Ampholine PAG plates and found few alterations compared with the non-attenuated controls. When electrophoresed for esterase profile, conidia of *M. anisopliae* isolate V245 from sub-cultures with reduced virulence showed significant changes in protein profile (Chapter 3). In contrast, isoelectrofocusing techniques used in this study showed that sub-culturing on artificial media did not change banding profile of water-extracted proteins but brought significant quantitative changes. This indicates that repeated sub-culturing may produce multifarious biochemical alterations, which influence pathogen virulence and thus may interfere with the whole process of pathogenesis.

There was no clear link between sub-culturing, virulence and Pr1 production. Even the most attenuated sub-cultures such as 3 and 9 of V245; 3 and 7 of V208 and 3 and 5 of V234 produced high quantities of cuticle-degrading protease Pr1 (Table 4.4) in nutrient poor MM media supplemented with aphid homogenate. Similarly, MM containing 1% Yeast Extract and beetle (*Phaedon cochleariae*) homogenate, induced similar Pr1 production in all tested sub-cultures of *M. anisopliae* isolate suggesting that repeated

sub-culturing had no affect on Prl production (Uribe, 1995). However, when non-virulent form of *B. bassiana* strain (which has lost its virulence towards *Galleria mellonela* during long term-storage at 4 °C) was compared with protease activity of the virulent form (which was obtained after several host passages) in casein medium, the capacity to release protease differed remarkably (Vilcinskas & Wedde, 1997). This difference diminished in Sabouraud medium suggesting that nutrient levels do regulate the expression of gene products of entomopathogenic fungi that are required for enzymatic digestion of host proteins (St Leger *et al.*, 1992). But these products (e.g. Prl) are not necessarily virulence determinants as suggested by St Leger *et al.* (1988) since sub-cultures with depressed Prl activities also cause high mortalities in mustard beetles (Uribe, 1995). The induction/repression responses of *M. anisopliae* isolate were similar for all sub-cultures studied indicating that regulatory controls at least for Prl protease were not affected by sub-culturing (Uribe, 1995).

Indeed, nested PCR reaction resulted in the similar 1.2 kb Prl PCR product for all sub-cultures of all three isolates of *M. anisopliae* studied in the present study. It is unlikely, however, that each sub-culture might have expressed *prl* gene of different type (St Leger *et al.*, 1994a) since repeated sub-culturing did not induce isoforms of Prl in *M. anisopliae* isolates (Uribe, 1995).

In summary, the results of this study have identified that continued *in vitro* inoculum production influences the level of virulence through changes of *M. anisopliae* attributes such as LC_{50} and LT_{50} values. Attenuation of virulence was always associated with inability of the fungus to cause rapid insect death at relatively low doses. Although all

isolates of *M. anisopliae* and their subsequent sub-cultures examined in this study produced Pr1, the link between this enzyme and fungal virulence has not been found.

CHAPTER 5

The effects of successive *in vitro* sub-culturing on physiological state of *Metarhizium anisopliae* conidia and its relation to virulence

5.1 INTRODUCTION

Important technological advances in the commercialisation of many mycoinsecticides have been made over the past ten years. As a result of these achievements, over 30 registered mycoinsecticide products have now been developed worldwide (Wraight *et al.* 2001). The active ingredient of almost all (89%) *Metarhizium anisopliae* formulations commercially available to-date is aerial conidia. This is because the pathogen produces large quantities of small hydrophobic conidia in dense masses. Strong and thick walls of aerial conidia not only confer environmental stability but also contribute to production efficiency and long-term storage stability (Wraight *et al.* 2001). Moreover, surface properties of aerial conidia determine how these reproductive propagules interact with biotic and abiotic factors, and their particular characteristics during dormancy, dispersal and their eventual association with a suitable substrate or host. Before a fungal spore can even launch an attack on the host cuticle, it must possess non-specific properties such as surface hydrophobicity (Fargues, 1984) and more specific linkages such as lectins (Boucias & Latgè, 1986) or antigens (Rath *et al.*, 1995) which allow for a more permanent binding to the insect cuticle to occur.

Previous studies have demonstrated that hydrophobin genes (*Sc3, Sc4, Sc1*) were abundantly expressed only at the time of hyphal (aerial) growth and formation of fruiting structures in *Schizophyllum commune*, suggesting an important role of hydrophobins in spore dispersal (Mulder & Wessels 1986; Schuren *et al.*, 1993). Hydrophobin is secreted as monomers with hydrophilic/hydrophobic interfaces that self-assemble at water-air and water-oil interfaces and also at interfaces between water and solid surfaces producing an

insoluble complex in a form of hydrophobic rodlet layer. The ability for interfacial self-assembly suggests that this layer interacts with adhesion to plant/insect surfaces. Wessels (1994) suggested that as a hypha grows over the surface, it secretes hydrophobins that self-assemble, not only at the side of the hypha facing air, but also at the side facing a solid hydrophobic surface and thus firmly glue two incompatible surfaces together.

Rodlet layers are common features of aerial hyphae, mushroom caps and spore surfaces of many fungal species. Similar rodlets were found on thalli of many lichens (Honneger, 1991). These cysteine-rich, low molecular weight proteins are SDS-insoluble but extractable with formic or trifluoroacetic acid (Wessels, 1992). Although common, the role of rodlet protein layers in fungi is still unclear. Disruption of the *rodA* gene from *Aspergillus nidulans* (Stringer *et al.*, 1991) and the *EAS* gene from *Neurospora crassa* (Bell-Pederson *et al.*, 1992; Lauter *et al.*, 1992) produced mutants that were easily wettable and lacked the hydrophobic rodlet proteins. These findings suggested that rodlets of hyphal and spore surfaces are instrumental in generating surface hydrophobicity. However, when rodlet layer of conidia of *Beauveria bassiana* was removed with formic acid (Bidochka *et al.*, 1995), it had not only retained the ability to bind to insect cuticle, but also retained a hydrophobicity value of 85% as determined by a phase exclusion assay. Stringer & Timberlake (1995) have identified another hydrophobin (*DewA*) that was present in the cell walls of *A. nidulans* conidiospores. When the *DewA* gene was disrupted, the spores were wettable by a solution containing 0.2% SDS and 50 mM ethylenediamine tetraacetic acid (EDTA). After disruption of both *RodA* and *DewA,* spores became more hydrophilic compared to those from mutants without the *RodA* gene.

St Leger *et al.* (1992) have found that during nutrient deprivation (transferring fungal cells from nutrient rich media (SDB) to nutrient poor media (minimal media)), *Metarhizium anisopliae* not only produced Pr1 transcript in less than 2 hr, reaching a maximum concentration after 24 hr, but also abundantly transcribed the hydrophobin gene *ssgA*. This gene was also highly expressed during appressoria formation *in vitro*. The authors suggested that ssgA peptide is involved in building the walls of these appressoria and could assist the hydrophobic attachment to cuticular surface and prevent desiccation. When sequenced, *ssgA* showed 48%, 64% and 67% identity to *Sc1, Sc3, S4* hydrophobins respectively, and 48% similarity to a rodlet protein from *Aspergillus nidulans*. This gene also showed a close resemblance to a wide range of toxins and agglutinins by their secondary structure. Similar findings were reported by Tabolt *et al.* (1993) in *Magnaporthe grisea* where a high transcriptional activity of hydrophobin gene *MPG1* was detected during appressorium formation, while a second peak of the same gene occurred during symptom development. The late expression of *MPG1* mRNA during disease development was suggested to relate to a phytotoxic effect, similar to that for cerato-ulmin in Dutch elm disease (Tabolt *et al.* 1993). Parallel findings of hydrophobins associated with infection structure formation in both plant and insect pathogens imply that hydrophobins may commonly be used for this purpose by fungal pathogens.

The ability to manufacture a BCA on an industrial scale and achieve a reliable end product that consistently performs well under field conditions is often regarded as the greatest problem hindering the development of commercial mycoinsecticide. Repeated *in vitro* culture is known to attenuate virulence and thus affects quality of the active ingredient. In order to resolve this problem, great efforts have been made to find ways of

preserving pathogen virulence (Schaerffernberg, 1964; Lane *et al.*, 1991b; Fargues & Roberts, 1983; Morrow *et al.*, 1989; Prenerova, 1994) or enhancing virulence (Lane *et al.*, 1991a; Hallworth & Magan, 1994c, 1995; Ibrahim *et al.*, 2002). However, the most common solution to this problem still involves storage of large quantities of inoculum passaged directly through a host or limitation of numbers of *in vitro* passages.

Many recent studies have demonstrated that ecophysiological manipulation has enormous potential as a strategy to reliably produce ecologically fit inocula through manipulation of endogenous reserves (Hallsworth & Magan, 1994a, 1994b; Pascual *et al.*, 1996; Jackson *et al.*, 1997; Frey & Magan, 1998; Teixido *et al.*, 1998a, 1998b; Anderson, 2000). However, exactly how endogenous reserves of modified organism relate to virulence still remains unknown.

Results, previously presented in Chapters 3 and 4, have clearly shown that continued sub-culturing of *M. anisopliae* on artificial growth media significantly influenced the ability of conidia of the entomopathogen to adhere, germinate and produce appressoria on the cuticle of *Myzus persicae*. The aim of this study was to (i) investigate the effect of repeated sub-culturing of *M. anisopliae* on artificial media on the assimilation of carbon substrates and the accumulation of endogenous reserves by the fungus and (ii) identify endogenous components that may account for virulence.

5.2 MATERIALS AND METHODS

5.2.1 Assimilation of carbon substrates

In order to determine the assimilation of carbon substrates by sub-cultures with contrasting LT_{50} values of the three isolates of *M. anisopliae*, API 50 CH strips (bioMérieux Vitek, Inc) were used. Each strip consisted of 50 microtubes containing a defined quantity of dehydrated substrate belonging to the carbohydrate family and its derivatives (heterosides, polyalcohols and uronic acids). Assimilation was indicated by growth of tested isolates and their sub-cultures in the cupules, when the substrate was the only source of carbon present.

Conidia of V245 (sub-cultures 1,3,5 and 9), V208 (sub-cultures 1,3,7 and 9) and V234 (sub-cultures 1,3,7 and 11) grown on SDA, were harvested and suspended in 0.03% Tween 80. One ml of conidial suspension (10^7 conidia ml^{-1}) was dispensed onto Petri dishes containing 15 ml of SDA. After 24 hr of incubation at 23°C in dark, sporelings were harvested by flooding the plates with 10 ml 0.03% Tween 80 solution, dislodged by agitation with a glass rod and washed three times before final re-suspension in distilled water (10^5 cfu ml^{-1}). The test procedure was performed in accordance with the manufacturer's recommendations.

5.2.2 Total Carbon, Sulphur and Nitrogen analysis

Aerial conidia were harvested from sub-cultures 1, 3, 5, 7, 9 and 11 for each isolate grown on both SDA and MM by gently scraping the surface of a culture plate into Water

G Chromasolv® containing 0.03% Tween 80. Suspended conidia were filtered twice through four layers of muslin to remove hyphal fragments before being washed in Water G three times by centrifugation at 3800 r.p.m for 20 min. The resultant spore pellets were frozen at –80 °C before being lyophilised in an Edwards Modulyo freeze dryer. Freeze-dried conidia were then assessed for carbon, sulphur and nitrogen content using a SC-144DR Carbon/Sulfur Analyser and a FP-528 Protein/Nitrogen Analyser, respectively (LECO Corporation, USA).

The SC-144DR Carbon/Sulfur Analyser is a software controlled instrument designed to measure the carbon and sulphur content in a wide variety of materials. Prior to the beginning of the sample analyses, a series of analyses is performed using blanks and calibration standards of various weight levels to establish an appropriate calibration curve. A sample is weighed into a combustion boat and analysis begins when the sample is placed in a pure oxygen environment typically regulated at 1350 °C (Fig. 5.1). The combination of furnace temperature and analyse flow causes the sample to combust. All sample material contained in the combustion boat goes through an oxidative-reaction process that causes carbon- and sulphur-bearing compounds to break down and free the carbon and sulphur. Carbon and sulphur are then oxidised to form CO_2 and SO_2, respectively. The design of the combustion system prevents the atmosphere from entering the combustion zone. Both sample gases are first swept through the boat stop to the back of the inner combustion tube, then forward between the inner and outer tube, allowing the sample gases to remain in the high temperature zone for a longer period and permit efficient oxidation (Fig.5.1). From the combustion system, the gases flow through two Anhydrone tubes to remove moisture, through a flow controller, which sets the flow of sample gases to 3.5 l min^{-1}, and then through the infrared detection cell. The carbon IR

cell measures the concentration of carbon dioxide gas. The sulphur IR cell measures the concentration of sulphur dioxide gas. The detector responds to the energy changes between the carrier gas and the sample gas and ultimately determines the concentration of carbon or sulphur by infrared spectrometry. As the analysis begin, the cell output decreases proportionally with the amount of carbon (as CO_2) and sulphur (as SO_2) in the IR cells. The computer reads the cell output nine times a second by an interval linearisation equation (Carbon or sulphur = (b)(area) + a, where b = software generated coefficient that indicates the slope of the line; a = the blank intercept) and then converts these values to a percentage/ppm value, using an equation present in the software which takes into account the sample weight, calibration and known moisture value.

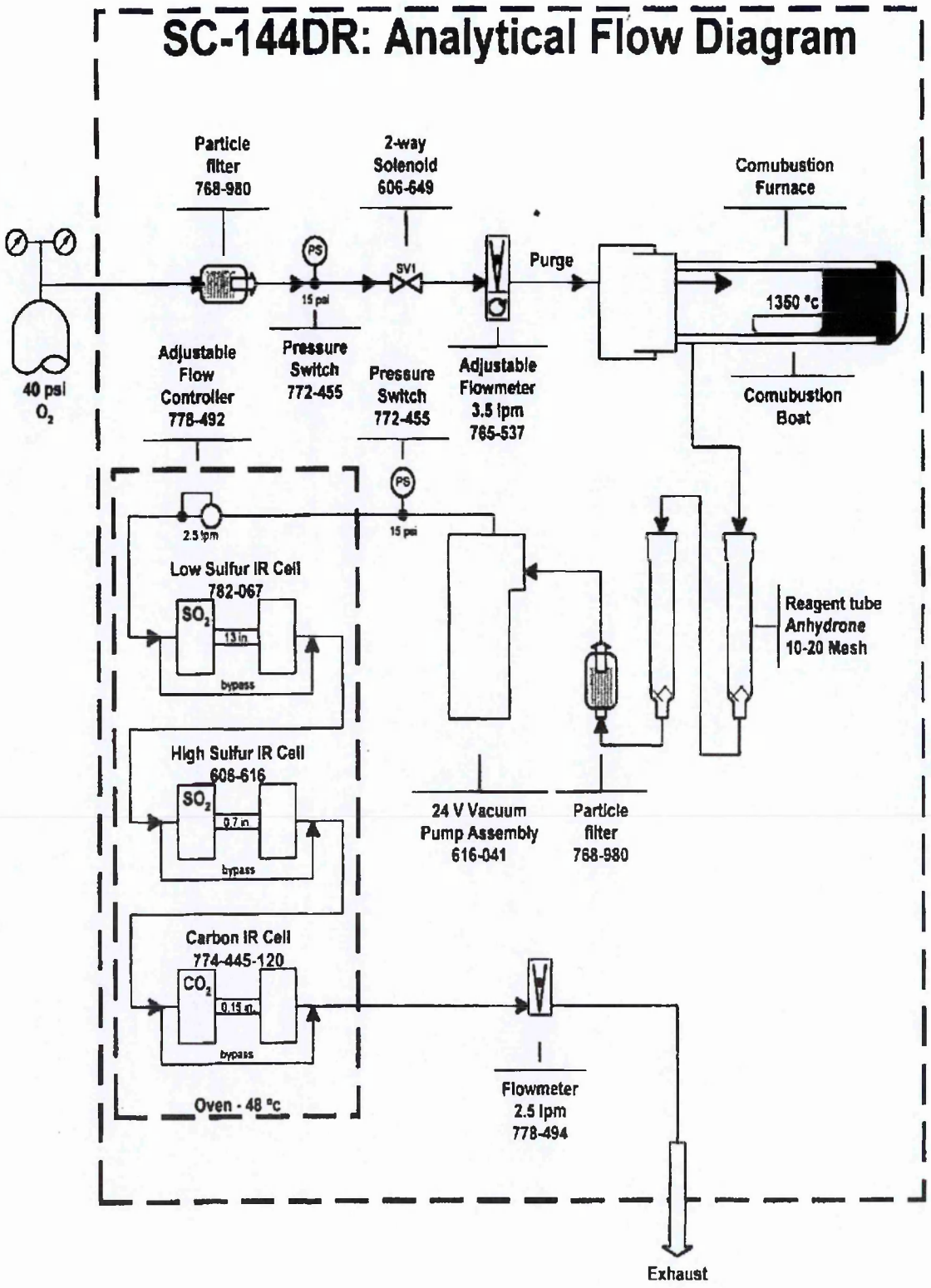


Figure 5.1. Analytical flow diagram of SC-144DR Carbon/Sulfur Analyser (from Manual, LECO Corporation, USA).

The FP-528 Protein/Nitrogen Analyser is a very similar instrument to the SC-144DR Analyser. During an Analyse cycle sample undergoes three phases: purge, burn and analyse (Fig.5.2). In the "sample-drop-purge" phase, the samples are weighed and then encapsulated in foil, placed in the loading head, sealed, and later purged of any atmospheric gases that have entered during sample loading. The ballast volume (zero volume at this point) and gas lines are also purged. During the second "burn" phase the samples are dropped into a hot furnace (850 °C) and flushed with pure oxygen for very rapid combustion. The products of the combustion (CO_2, H_2O, NO_x and N_2) are then passed through the furnace filter and thermoelectric cooler, and finally collected in the ballast volume (Fig.5.2). In the third and last "analyse" phase, the combustion gasses in the ballast are allowed to become homogenous by passive mixing. A 3 ml aliquot is captured in a loop before the free-floating ballast piston is forced down to evacuate the ballast. The samples are then swept through hot copper to remove oxygen and change NO_x to N_2, then through Lecosorb and Anhydrone to remove CO_2 and H_2O, respectively. The remaining combustion product, N_2 in a helium carrier, is measured by the thermal conductivity cell. A precooler and thermoelectric cooler in the flow path, after the furnace, removes moisture before it can enter the ballast. The final result is displayed as weight percentage of nitrogen (or protein if selected).

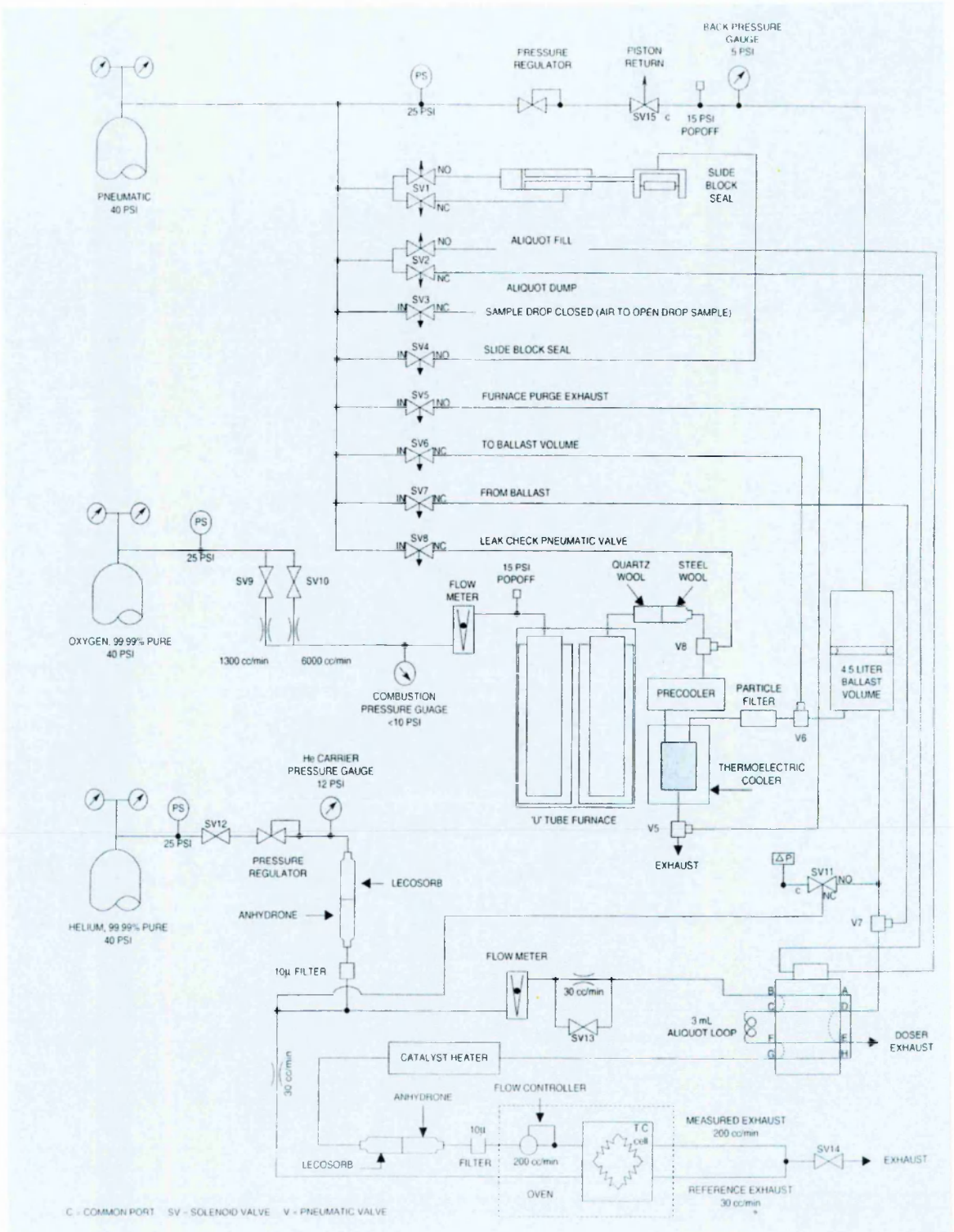


Figure 5.2. Analytical flow diagram of FP-528 Protein/Nitrogen Analyser (from Manual, LECO Corporation, USA).

Two hundred mg of freeze-dried conidia of each sub-culture for all three isolates tested were loaded into the analysers and then subjected to analytical procedures described above. The SC-144DR Carbon/Sulfur and FP-528 Protein/Nitrogen Analysers were calibrated using $CaCO_3$ (12% carbon)/bituminious coal (1.18% sulphur) as carbon/sulphur standards and EDTA 502-092 (carbon 40.97%, Hydrogen 5.97% and Nitrogen 9.57%) as protein/nitrogen standard, respectively, (LECO Corporation, USA).

5.2.3 Polyols and trehalose analysis

Conidia from sub-cultures 1, 3, 5, 7, 9 and 11 for each isolate grown on SDA medium and conidia from sub-culture 1 for all three isolates grown on MM were harvested and freeze-dried using the methods described in previous section. Freeze-dried conidia were then processed and analysed for polyols (mannitol, arabitol, erythritol and glycerol) and trehalose content using the protocol described in Section 2.2.7.

5.2.4 Extraction, quantification and characterisation of conidial surface proteins

The surface proteins of conidia from sub-cultures 1, 3, 5, 7, 9 and 11 for each isolate grown on SDA were studied, with interest concentrating on isolating hot-SDS-insoluble, formic acid (FA) soluble proteins, that may convey hydrophobic properties to conidial surfaces. Usually, the proteinaceous rodlets of hydrophobic conidia are very resilient and can only be disrupted with a high-powered sonicator and/or by harsh chemical treatments involving very concentrated organic acids (Wessels *et al.,* 1991). For this purpose, aerial conidia were harvested, washed, filtered and lyophilised as described in Section 2.2.7. To obtain total SDS-soluble proteins, the freeze-dried conidia were weighed into 2 ml

microtubes and subjected to 1% SDS extraction buffer (1% (w/v) SDS in 0.05M Tris-HCl (pH 8.0); 1 mM EDTA and 100 μg PMSF) at 100 °C for 10 min. The extraction procedure was repeated four times with the resultant supernatant being collected and frozen at –20 °C for further analysis. SDS-extracted conidia were washed twice with water and then subjected to 96% (v/v) FA (100 μl g [dry wt]$^{-1}$ in a sonicating water bath (Science Exchange Service, UK) for 2 hr, keeping the temperature below 10 °C. After centrifugation (12 000 r.p.m. for 10 min), 2 volumes of performic acid (PA) were added to the supernatant, and oxidation was allowed to proceed on ice for 4 hr. This treatment oxidises cystine to cysteic acid, breaking disulfide bridges, and thereby permanently breaks the hydrophobin structure leaving the monomers intact and biologically active. Periodic acid was prepared by mixing one volume of 30% H_2O_2 with 9 volumes of 96% FA and used after standing at room temperature for 1 hr. Formic acid extracts, before and after oxidation with PA, were diluted 20 times with water, freeze-dried and stored at –20 °C till needed.

Total SDS-soluble and formic/performic acid soluble proteins were estimated using Bradford dye-binding assay (see Section 2.2.8). While freeze-dried formic/performic acid soluble proteins were dissolved in Tris-glycine sample buffer, the SDS extracted proteins were diluted two fold with the same buffer, which comprised of the following: 62.5 mM Tris-HCl (pH 6.8); 10% (v/w) glycerol; 2% (w/v) SDS; 125 mM DTT and 0.005% (w/v) bromophenol blue. Samples were then heated with the sample buffer at 95 °C for 5 min and aliquots containing 2-15 μg protein were then loaded on Tris-glycine gels with 5% stacking and 15% resolving gels prepared according to Bio-Rad's standard protocol. All gels were run at 100 V for the first 40 min and then at increased to 150 V for the rest of running time. After electrophoresis, protein bands were stained with either coomassie

blue (see Section 2.2.7) or silver staining (based on method of Oakley *et al.*, 1980). Since silver stain is extremely sensitive to contaminating proteins, it is important to use acid-clean glassware and pure reagents. Slab gels were washed in 500-1000 ml distilled water twice before adding a freshly made ammoniacal silver solution. To prepare 100 ml of the solution 1.4 ml of fresh NH_3 was added to 21 ml of 0.36% NaOH. While vigorously agitating, 20 ml of freshly made 5% (w/v) $AgNO_3$ were added to the solution. When transient brown precipitate had cleared the solution was then topped up with distilled water to the final volume of 100 ml. To ensure even staining and to prevent sticking of the gel to the staining container, 200 ml of silver solution were added to each slab gel agitating in 20 x 20 cm container. To prevent silver deposition on the surface of the gel, the slab gel was stained for no longer than 15 min in silver solution and then removed to a new clean container containing enough distilled water to allow the gel to float freely. Following 2 min wash, the gel was transferred to a freshly prepared developer containing 0.005% (w/v) citric acid and 0.019% (v/v) formaldehyde (made by dilution of 38% formaldehyde solution containing 10-15% methanol). When a dark background begun to develop, the gel was removed from the solution and washed for at least 1 hr in distilled water with agitation and three changes of water. After washing, the gel was fixed in 7% acetic acid.

The integrated use of fluorescent microscopy and calcofluor staining (Chapter 2, Section 2.2.6), were also used to determine if *in vitro* sub-culturing affected carbohydrate composition (e.g. ß-glucans) at the surface of the conidial cell wall of attenuated and non-attenuated conidia, as well as conidia collected directly from aphid cadavers.

5.3 RESULTS

5.3.1 Carbohydrate utilisation by virulent and attenuated conidia of three *M. anisopliae* isolates

The API 50 CH test was semi-quantified as follows: 0 was given to a negative reaction, where known carbohydrate inhibited the hyphal growth and showed no change in colour. Values 1 and 2 were given to a weak reaction, where known carbohydrate stimulated very slow growth and indicated a slight change in colony colour. Values 3 and 4 were given to intermediate reactions, and a value of 5 given to a maximum reaction, where known carbohydrates stimulated good germination of conidia and intensive hyphal growth, changing colony colour to a maximum intensity of value 5.

The API 50 CH profiles of the isolates are presented in Table 5.1. In general, all sub-cultures of V245 tested were able to utilise more than twenty substrates, whereas sub-cultures of V234 showed reaction to very few substrates tested. Isolate V208 was regarded as an isolate of intermediate reaction ($F_{2,292} = 662.54$, $P < 0.001$).

The most common carbohydrates readily utilised by sub-culture 1 of all three isolates, were adonitol, D-mannose, L-sorbose, mannitol, sorbitol, esculine and D-arabitol, sorbitol, esculine and D-arabitol. Some substrates including β methyl-xyloside, α methyl-D-glucoside, inuline, gluconate and 5-ceto-gluconate, inhibited the hyphal growth of sub-culture 1 of all three isolates tested. Repeated sub-culturing clearly affected carbon utilisation profile of all three isolates ($F_{48,292} = 174.67$, $P < 0.001$). For example, sub-culture 1 of isolate V245 was not able to utilise inositol or glycogen, but this ability was

Table 5.1 Carbon source utilisation profiles of *M. anisopliae* isolates as determined on API 50 CH strips following successive sub-culturing on SDA media

[a] Each value represents the mean of three replicates and given to a reaction for each sub-culture, where 0 is given to a negative reaction when known carbohydrate inhibits the hyphal growth and shows no change in colony colour. Values 1and 2 are given to a weak reaction, where known carbohydrate stimulates slow growth and indicates a slight change in colony colour. Values 3 and 4 are given to intermediate reactions, and a value of 5 is given to a maximum reaction, where known carbohydrates, stimulate germination of conidia and intensive hyphal growth, changing colony colour to a maximum intensity of value 5.

Substrate	V245				V208				V234			
	Subculture											
	1	3	5	9	1	3	7	9	1	3	7	11
Control	0[a]	0	0	0	0	0	0	0	0	0	0	0
Glycerol	5	5	4	5	2	3	3	3	3	3	3	2
Erythritol	1	1	0	1	1	0	0	0	2	0	0	0
D-Arabinose	2	3	2	2	2	2	2	2	2	1	2	0
L-Arabinose	2	5	4	3	2	3	2	2	2	3	3	2
Ribose	2	4	4	4	1	3	4	2	2	3	3	2
D-Xylose	3	5	4	5	1	3	5	4	1	3	3	2
L-Xylose	1	0	0	0	0	0	0	0	2	0	2	0
Adonitol	4	5	5	5	5	3	2	3	3	4	3	2
β-Methyl-xyloside	0	0	0	0	0	0	0	0	0	0	0	0
Galactose	5	0	0	2	3	3	3	3	2	2	3	0
D-Glucose	2	5	4	5	0	3	3	3	3	3	3	5
D-Fructose	5	3	4	2	1	2	2	3	2	3	3	0
D-Mannose	5	5	5	5	5	5	5	3	2	3	3	1
L-Sorbose	5	2	2	2	5	5	5	3	2	2	3	1
Rhamnose	0	0	0	2	0	0	0	0	2	3	3	0
Dulcitol	0	0	0	0	3	2	0	1	2	2	2	0
Inositol	0	3	3	3	0	2	2	2	2	2	2	2
Mannitol	5	3	3	3	5	3	3	3	2	2	3	0
Sorbitol	4	3	3	3	5	3	3	3	2	0	3	0
α–Methyl-D-mannoside	0	0	0	0	1	0	0	0	0	2	3	0
α–Methyl-D-glucose	0	0	0	0	0	0	1	1	0	3	1	0
N-acetyl-glucosamine	1	4	5	5	0	3	4	2	0	3	1	0
Amygdaline	2	1	0	1	0	3	4	2	0	4	2	0
Arbutine	2	2	2	3	0	0	2	0	0	0	2	0
Esculine	5	5	5	5	5	2	3	3	3	1	0	0
Salicine	3	1	0	0	0	0	2	1	0	3	0	0
Cellobiose	2	3	2	3	1	3	3	2	0	2	0	0
Maltose	3	3	4	3	0	3	3	2	1	2	2	0
Lactose	1	3	0	1	0	2	0	0	1	2	2	0
Melibiose	2	2	4	2	0	2	2	1	2	3	2	0
Saccharose	4	4	4	4	1	4	5	2	2	3	2	0
Trehalose	4	5	4	4	1	4	5	2	0	0	2	0
Inuline	0	0	0	0	0	0	0	0	0	3	0	0
Melezitose	1	3	3	2	0	1	2	2	0	3	2	0
D-Raffinose	1	3	3	2	3	3	4	2	0	3	3	2
Amidon	2	5	3	4	2	5	5	5	2	4	3	5
Glycogen	1	5	5	5	2	4	4	4	2	2	5	0
Xylitol	1	3	0	2	2	0	4	3	3	3	3	0
β–Gentiobiose	3	3	3	2	2	3	4	3	0	2	2	0
D-Turanose	3	3	3	2	1	2	2	3	0	3	2	0
D-Lyxose	5	3	3	2	1	2	2	3	0	3	3	0
D-Taragatose	0	2	0	0	2	0	0	0	0	0	1	0
D-Fucose	0	0	0	0	2	0	2	0	0	0	2	3
L-Fucose	0	4	0	2	3	2	3	3	0	2	2	3
D-Arabitol	5	5	4	5	5	3	3	4	2	4	5	0
L-Arabitol	5	4	2	4	0	3	3	3	1	4	3	0
Gluconate	0	0	0	0	0	0	1	2	0	0	0	0
2-ceto-gluconate	0	2	0	2	0	2	2	2	3	2	3	1
5 ceto-gluconate	0	2	4	3	0	2	3	2	0	3	4	1

LSD

Isol 0.067 ($P<0.001$); Subc 0.272 ($P<0.001$); Med.Isol 0.471 ($P<0.001$)

improved by only one passage through SDA. The ability of V208 to assimilate carbohydrates such as glucose, N-acetyl-glucosamine, amygdaline, saccharose, trehalose, amidon, glycogen and L-arabitol, was enhanced by three passages on SDA culture media. Although better absorption of numerous carbohydrates (Table 5.1) by isolate V234 was achieved by sub-culture 3, sub-culture 11 lost its ability to absorb most of the carbohydrates tested with the exception of glucose, amidon, D-fucose and L-fucose. Even though there was no obvious link established between carbohydrate preferences and virulence, the general inclination of attenuated sub-cultures (sub-cultures with higher LT_{50} values) to in-take the easy-utilisable substrates was noticed.

5.3.2 Accumulation of endogenous carbon, nitrogen and sulphur in virulent and attenuated conidia of three *M. anisopliae* isolates

The observations recorded above suggest that repeated sub-culturing on SDA media could either increase or reduce sugar utilisation and thus affect the accumulation of endogenous carbon, nitrogen and sulphur. To test whether C:N ratio of attenuated sub-cultures differed from that of virulent forms, carbon, sulphur and nitrogen were quantified using SC-144DR Carbon/Sulfur and FP-528 Protein/Nitrogen analysers, respectively. Data presented in Table 5.2 shows that *in vitro* sub-culturing exerted an influence on total carbon, nitrogen and sulphur content for all inocula tested, regardless of the culture media used. In general, sub-cultures with higher LT_{50} values tended to accumulate more endogenous carbon and less nitrogen, resulting in a high C:N ratio. For example, attenuated sub-culture 3 of V208 (LT_{50} value of 6.1) accumulated 480.8 mg g^{-1} of endogenous carbon and 53.4 mg g^{-1} of endogenous nitrogen, representing a

C:N ratio of 9.0. Conversely, the aggressive form of V208 inocula (sub-culture 1 with LT_{50} value of 3.6) accumulated less carbon (450.8 mg g^{-1}) and more nitrogen (76.1mg g^{-1}), giving a C:N ratio of 5.9. Furthermore, regression analysis revealed a significant and strong positive linear relationship between lethal time (LT_{50}) and endogenous C:N ratio for sub-cultures of isolate V234 ($y=4.62+0.33x$; $r^2 = 0.81$; $P < 0.05$). Although not significant, similar trends were also noticed for sub-cultures of isolates V245 ($r^2 = 0.58$; $P = 0.07$) and V208 ($r^2 = 0.47$; $P = 0.13$). For inocula passaged through MM media, the relationship between virulence and C:N ratio was highly significant ($r^2 = 0.78$; $P < 0.001$). Moreover, when C:N ratios of conidia were greater than 6:1, germination of all sub-cultures of all three isolates decreased with increasing endogenous C:N ratios ($y=10.22-0.042x$; $r^2 = 0.56$; $P < 0.001$).

Conidia produced on SDA contained more sulphur (5.31 mg g^{-1}) than those grown on MM (5.16 mg g^{-1}). Sulphur content was also greatly affected by repeated sub-culturing and depended on the isolate tested and medium used for sub-culturing ($P<0.001$) (Table 5.2). In addition, when isolates were cultured on SDA, it was noted that sub-cultures with higher nitrogen content also contained higher quantities of endogenous sulphur ($r^2 = 0.55$; df=1,16; $P < 0.001$). In contrast, an inverse relationship existed between nitrogen and sulphur contents of inocula produced on MM medium ($r^2 = 0.74$; df = 1,16; $P < 0.001$).

Table 5.2. Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA and MM media on the accumulation of total carbon, nitrogen (C:N) and sulphur in conidia.

Isolate	Sub-culture	Carbon mg g^{-1}		Nitrogen mg g^{-1}		C:N		Sulphur mg g^{-1}	
		SDA	MM	SDA	MM	SDA	MM	SDA	MM
V245	1	455.6	430.7	79.9	106.8	5.7	4.0	6.0	4.7
	3	474.2	434.8	75.1	110.5	6.3	3.9	6.0	4.8
	5	473.0	446.8	77.0	103.5	6.1	4.3	6.5	4.6
	7	469.3	435.6	78.6	93.7	6.0	4.6	6.5	4.2
	9	476.7	452.7	75.2	100.5	6.3	4.5	6.2	4.4
	11	467.6	435.6	79.5	100.9	5.9	4.3	6.4	4.8
V208	1	450.8	469.9	76.1	106.4	5.9	4.4	3.5	4.5
	3	480.8	457.2	53.4	102.8	9.0	4.4	3.6	4.5
	5	482.0	460.8	57.3	106.3	8.4	4.3	5.1	4.5
	7	476.6	450.9	59.6	100.0	8.0	4.5	3.9	4.8
	9	479.3	464.2	53.8	97.5	8.9	4.8	3.5	4.7
	11	486.3	448.9	56.8	99.9	8.6	4.5	4.5	4.6
V234	1	502.5	408.2	83.2	87.7	6.0	4.7	6.0	6.3
	3	478.0	413.5	74.4	82.1	6.0	5.1	5.9	6.2
	5	469.4	411.3	74.0	87.3	6.3	4.7	5.6	6.3
	7	455.0	410.9	79.5	84.8	5.7	4.9	5.6	6.2
	9	466.2	420.7	76.6	83.7	6.1	5.0	5.1	6.5
	11	467.5	406.3	75.7	86.1	6.2	4.7	5.8	6.2
LSD	Med	2.00		0.77		0.04		0.11	P <0.01
	Isol	2.44		0.94		0.05		0.14	P <0.001
	Subc	3.46		1.34		0.07		0.19	P <0.01
	Med.Isol	3.46		1.34		0.07		0.19	P <0.001
	Med.Subc	4.89		1.89		0.10		0.27	P <0.001
	Isol.Subc	5.99		2.31		0.12		0.33	P <0.05
	Med.Isol.Subc	8.46		3.27		0.17		0.47	P <0.001
	P	<0.001		<0.001		<0.001		<0.001	

5.3.3 Accumulation of endogenous polyols and trehalose in virulent and attenuated conidia of three *M. anisopliae* isolates

The quantities of polyhydroxy alcohols (polyols) extracted from conidia of *M. anisopliae* produced on SDA, significantly differed depending on the isolate or sub-culture tested (Table 5.3). Accumulation of the endogenous polyols also depended on the response of individual isolates to repeated sub-culturing. No trehalose was detected in any of the sub-cultures tested for all three isolates cultivated on SDA. Very small quantities of trehalose, however, were detected in conidia produced on MM medium (Table 5.4). Sub-cultures of V234 always contained more glycerol (102.0 mg g^{-1}) and mannitol (75.2 mg g^{-1}) than sub-cultures of V208 (68.8 mg g^{-1} and 50.7 mg g^{-1}, respectively) or V245 (63.0 mg g^{-1} and 21.1 mg g^{-1}, respectively). Erythritol content of conidia produced on SDA media was very similar for all three isolates tested ($P>0.05$) (Table 5.3). Sub-cultures that germinated poorly on SDA medium (Table 3.1) and had low LT_{50} values (Figure 4.3), also generated higher amounts of glycerol, mannitol and traces of arabitol. Conversely, most of the attenuated inocula from all three isolates contained less erythritol than non-attenuated inocula (Table 5.3). When combined, total polyol content of conidia for all sub-cultures was shown to be strongly related to the virulence of the same conidia ($r^2 = 0.50; F_{1,16}=16.036$; $P <0.001$). Conidia from sub-culture 1 of all three isolates grown on MM, contained erythritol, arabitol and small quantities of mannitol and trehalose (Table 5.4).

Table 5.3. Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA on the accumulation of endogenous polyols (mg g^{-1} dry weight) in conidia.

Isolate	Sub-culture	Polyol content			
		Glycerol mg g^{-1}[a]	Erythritol mg g^{-1}	Mannitol mg g^{-1}	Arabitol mg g^{-1}
V245	1	29.0	49.8	3.5	0.0
	3	108.0	42.7	11.3	1.0
	5	72.6	30.2	2.8	0.0
	7	51.1	27.6	23.6	0.0
	9	66.7	22.7	65.3	0.5
	11	50.6	31.7	6.6	0.0
V208	1	16.6	52.7	40.5	0.0
	3	74.5	43.6	125.9	0.0
	5	81.8	30.2	6.1	0.0
	7	74.4	49.9	41.9	0.0
	9	71.0	34.5	77.1	0.1
	11	94.2	20.4	25.3	0.0
V234	1	78.5	35.3	12.4	0.0
	3	111.0	29.6	199.1	0.0
	5	101.7	33.7	107.8	0.1
	7	87.2	36.6	11.9	0.0
	9	110.6	24.9	21.1	2.6
	11	122.9	31.6	131.3	0.0
LSD	Isol	10.3 ($P < 0.001$)	6.3 ($P > 0.05$)	9.8 ($P < 0.001$)	
	Subc	14.5 ($P < 0.001$)	8.8 ($P < 0.01$)	15.0 ($P < 0.001$)	
	Isol*Subc	25.25 ($P < 0.05$)	15.3 ($P < 0.05$)	26.0 ($P < 0.001$)	

[a]Each value represents the combined means of three experiments.

Table 5.4. Accumulation of endogenous polyols and trehalose (mg g^{-1} dry weight) in conidia of sub-culture 1 for three isolates of *Metarhizium anisopliae* cultured on MM medium.

Isolate	Erythritol mg g^{-1a}	Mannitol mg g^{-1}	Arabitol mg g^{-1}	Trehalose mg g^{-1}
V245	49.4	2.6	34.8	2.5
V208	75.0	2.9	38.2	8.9
V234	47.0	2.4	43.1	1.2
LSD	20.1 (P<0.05)	0.9 (P>0.05)	9.7 (P>0.05)	2.9 (P<0.05)

[a]Each value represents the combined means of three experiments.

5.3.4 Expression of surface proteins during continued *in vitro* sub-culturing

Analysis of hot-SDS-extracted proteins from conidial walls of *M. anisopliae* revealed a slight, but not significant difference, between the three isolates (Plate 5.1a-b). Repeated extractions of surface proteins with hot SDS resulted in a gradual decline in the amount of proteins extracted after each step (Plate 5.1a-b). The majority of the proteins (80%) were extracted from the first and second SDS treatments. Fourth SDS extraction yielded non-quantifiable amount of proteins, suggesting that all SDS-soluble proteins were successfully removed from conidial surfaces. SDS-treated conidia were then subjected to treatment with formic acid (FA) to extract hydrophobin-like surface proteins. FA-soluble proteins in conidial extracts were separated on Tris/Glycine gels of 15% acrylamide/bis since these gels adequately resolved the low molecular weight (MW) proteins of special interest (Plates 5.2a-b) as well as allowing for the identification of higher MW proteins (5.1a-b). All corresponding FA extracts possessed wide, defused single bands between 6.5 and 11 kDA (Plate 5.2a). Oxidation of FA

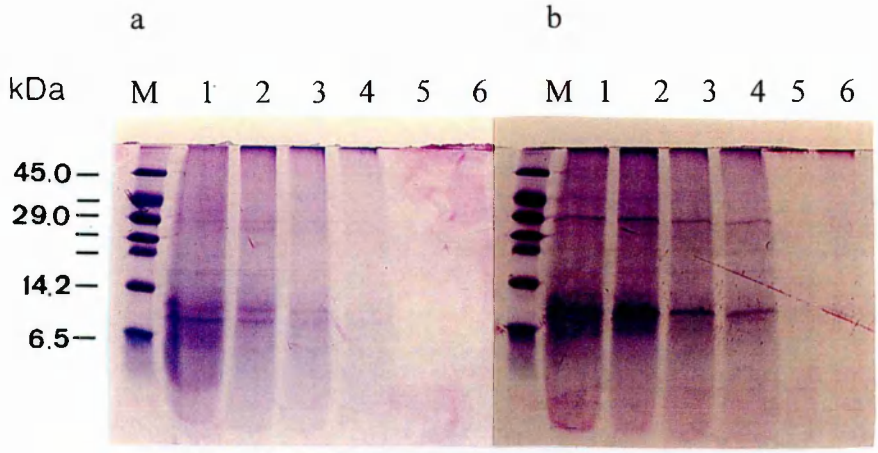


Plate 5.1a-b. SDS-PAGE protein profile of conidia of isolates V245 (a) and V208 (b) sub-cultured once on SDA media and treated with hot SDS once (lane1), twice (lane 2), three times (lane 3), four times (lane4) and then treated with formic acid (lane 5) and subsequently subjected to performic acid treatment (lane 6). M (protein markers).

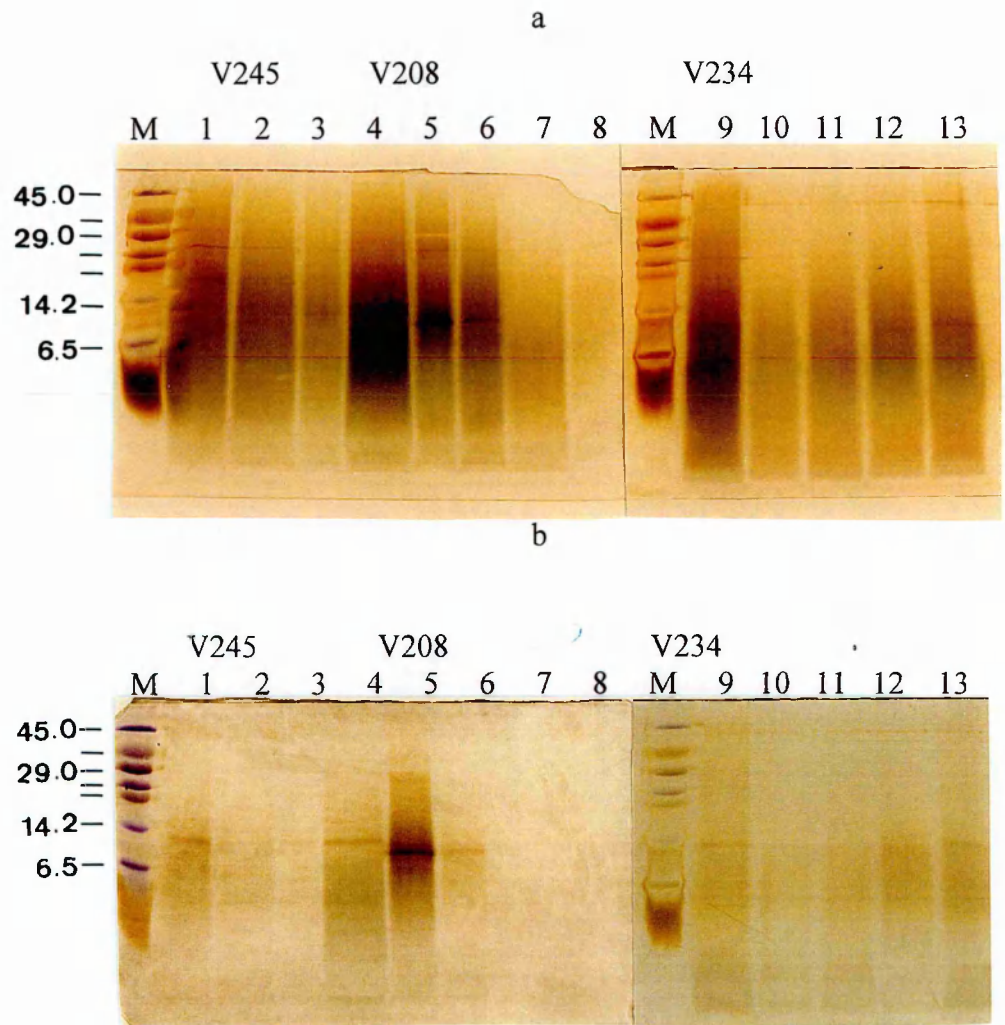


Plate 5.2a-b. SDS-PAGE of rodlet proteins dissolved in formic acid (a) from conidia harvested from sub-cultures 9 (lane 1), 7 (lane 2), 5 (lane 3), 3 (lane 4) of isolate V245; sub-cultures 7 (lane 5), 5 (lane 6), 3 (lane 7) and 1 (lane 8) of isolate V208 and sub-cultures 11 (lane 9), 9 (lane 10), 7 (lane 11), 5 (lane 12) and 3 (lane 13). (b) formic acid extracts (as in (a)) subjected to perfomic acid treatment. M (protein markers).

extracts with performic acid produced higher MW proteins of 12.5 kDA for sub-cultures of V208 isolate and 13.5 kDA proteins for sub-cultures of V245 and V234 isolates (Plate 5.2b). These bands also appeared to be significantly clearer and more prominent than bands for the FA extracts.

Table 5.5. Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA medium on the total amount of spore surface proteins extracted after sequential four treatments with SDS and one treatment with FA.

Isolate	Sub-culture	Total protein content, mg g^{-1} (SE)	
		SDS-soluble[a]	FA-soluble
V245	1	9.18 (1.44)	2.10 (0.31)
	3	13.94 (1.90)	4.09 (0.43)
	5	7.93 (1.42)	2.01 (0.22)
	7	9.89 (1.12)	2.89 (0.34)
	9	11.76 (1.22)	5.19 (0.36)
	11	8.84 (0.93)	2.15 (0.41)
V208	1	10.51 (0.14)	0.34 (0.02)
	3	16.32 (0.29)	0.68 (0.19)
	5	16.85 (0.17)	0.89 (0.10)
	7	16.73 (0.45)	3.91 (0.34)
	9	12.47 (0.55)	1.09 (0.22)
	11	21.01 (1.18)	0.13 (0.01)
V234	1	19.64 (2.21)	0.50 (0.13)
	3	20.56 (1.93)	2.84 (0.16)
	5	23.05 (1.46)	1.37 (0.21)
	7	18.37 (0.86)	1.18 (0.18)
	9	16.49 (1.01)	0.34 (0.09)
	11	18.63 (1.12)	0.68 (0.23)

[a]Each value represents the combined means of three experiments.

The sequential spore extraction protocol was shown to be successful in not only aiding the purification and resolution of spore hydrophobin-like proteins, but also yielded data relating to the effects of continued *in vitro* sub-culturing on the amounts of FA-soluble peptides polymerised on the surface of hydrophobic conidia (Table 5.5). For example, sub-cultures 3 and 9 of V245, sub-cultures 3, 5,7 and 11 of V208 and sub-cultures 3 and

5 of V234, possessed significantly more SDS-soluble surface proteins than sub-cultures 5 and 11 of V245 or 1 and 9 of V208 or sub-culture 9 of V234. Conidial FA-extracts of attenuated sub-cultures 3 and 9 of V245 isolate, sub-cultures 7 and 9 of V208 isolate and sub-cultures 3, 5 and 7 of V234 isolate also contained significantly more hydrophobin-like proteins than those of non-attenuated sub-cultures (Table 5.5). Sub-cultures which contained more FA-soluble proteins also showed protein bands of higher intensity (Plate 5.2b).

Calcofluor stained conidia fluoresced to different degrees depending on individual isolate ($F_{2,160} = 39.13$, $P < 0.001$), sub-culture ($F_{6,160} = 207.92$, $P < 0.001$) and their calcofluor binding capacity ($F_{12,160} = 30.70$, $P < 0.001$). For example, isolate V208 bound more calcofluor and fluoresced more intensely than conidia of isolates V245 and V234. Conidia from aphid cadavers, however, exhibited very low calcofluor binding affinities resulting in poor fluorescence (Table 5.6).

Table 5.6. Effect of successive sub-culturing of three isolates of *Metarhizium anisopliae* cultured on SDA medium on fluorescence intensity (arbitrary units) of conidia as determined by an LS 30 luminescence spectrometer operating at an excitation wavelength of 350 nm and an emission wavelength of 435 nm.

Sub-culture	Isolate		
	V245	V208	V234
1	91.0[a]	90.3	63.2
3	122.6	105.7	75.8
5	65.7	111.7	69.1
7	63.2	93.1	79.8
9	95.0	86.0	79.6
11	52.1	75.6	94.9
Insect	19.4	18.8	17.7
LSD			
Isol 1.19 (P <0.001)			
Subc 1.81 (P <0.001)			
Isol*Subc 3.15 (P <0.001)			

[a]Each value represents the combined means of three experiments.

5.4 DISCUSSION

When developing a commercial BCA, it is important to determine conditions that will provide optimal pathogenicity and sustain the agents' sporulation, virulence, host specificity and ecological fitness (Butt *et al.*, 2002). BCAs that are able to tolerate or withstand environmental stresses such as nutrient deprivation, low humidity and fluctuating temperatures will effectively establish and survive under fluctuating field conditions and thus improve their biocontrol potential. One way of improving ecological fitness of inocula is to modify its endogenous reserves (Teixidó *et al.*, 1998b). Culture media has been shown to influence the endogenous composition of fungal conidia (Fargues & Roberts, 1983; Lane *et al.*, 1991a; Jackson & Schisler, 1992; Yu *et al.*, 1998; Hallsworth & Magan, 1994b; Teixidó *et al.*, 1998b; Magan, 2001; Ibrahim *et al.*, 2002). For example, Hallsworth & Magan (1994b) demonstrated an increased accumulation of endogenous polyhydroxy alcohols (polyols) and trehalose in conidia of *Beauveria bassiana* and *Metarhizium anisopliae* produced on media with high concentrations of glycerol or trehalose. Similarly, endogenous lipids and carbohydrates, including glycogen, accumulated to significantly higher levels in blastospores of *B. bassiana* harvested from nitrogen-limited cultures than in blastospores harvested from carbon-limited cultures (Lane *et al.*, 1991a). These results are in agreement with those obtained from the study conducted in Chapter 2, where conidia of three isolates of *M. anisopliae* were shown to accumulate significantly higher levels of polyols and total proteins if grown on insect cuticles or nitrogen-limited medium (MM) than conidia cultured on yeast extract agar (YEA) or SDA media. The results obtained from this study have shown that conidia produced on SDA contained large amounts of glycerol but lacked trehalose (Table 5.3), while MM-grown inocula

(Table 5.4) contained no glycerol but accumulated higher quantities of arabitol and trehalose. Furthermore, continued sub-culturing of *M. anisopliae* on both SDA and MM media was shown to influence the ratio of endogenous compounds (e.g. C:N ratio, endogenous proteins, sugars and polyols) accumulated in resultant inocula. For example, conidial progeny of attenuated sub-cultures of all three isolates sub-cultured on SDA and MM media were found to contain more endogenous carbon and less nitrogen resulting in high C:N ratio. In contrast, the most virulent conidia directly collected from aphid cadavers (Table 2.7), conidia grown on MM media (Tables 2.7 and 5.2) and conidia sub-cultured once on SDA medium (sub-culture 1, Table 5.2) were shown to accumulate the highest amount of endogenous nitrogen.

High amounts of endogenous proteins in conidia with low C:N (C:N ratio < 6.0, Table 5.2) may provide the amino acid pool necessary for protein synthesis and thus facilitate rapid germination and frequent appressorium formation in *Metarhizium* isolates. Endogenous supplies of amino acids for *de novo* protein synthesis were shown to be essential for initial events of germination and differentiation in *Colletotrichum lagenarium*, *Metarhizium anisopliae*, *Glomerella magna* and *Colletotrichum truncatum*, while subsequent events, such as germ tube elongation, often used exogenous sources of carbon and nitrogen (Suzuki *et al*, 1981; St. Leger *et al*, 1989, Bhairi *et* al, 1990; Jackson & Schisler, 1992). Indeed, the results of the studies undertaken here have shown that C:N ratio of conidia of all three isolates repeatedly sub-cultured on SDA and the incidence of germination on the same media were linearly related ($r^2 = 0.56$; $P<0.001$) suggesting that frequent sub-culturing may alter the balance of endogenous carbon and nitrogen and thus influence the speed at which fungal pathogen are able to germinate. Moreover, since conidial C:N ratios of isolate V245 sub-cultured on SDA

and all three isolates sub-cultured on MM were significantly related (r^2 =0.58; P=0.07 and r^2 = 0.78; P<0.001, respectively) to virulence, determined by LT_{50} values, it would appear that C:N ratio influenced the virulence of conidia of *Metarhizium anisopliae* by influencing the speed of conidial germination.

Frequent sub-culturing on SDA medium has also been shown to affect the amounts of low and high molecular weight polyols and trehalose accumulated in conidia of *M. anisopliae* isolates used in this study. For example, conidia of attenuated sub-cultures contained five times more intracellular glycerol and 20 times more arabitol than conidia of non-attenuated sub-cultures (Tables 5.3). However, such changes were less apparent if the same isolates were repeatedly sub-cultured on nutrient poor MM media. Why continuous sub-culturing of *M. anisopliae* isolates on SDA medium caused greater instability than sub-culturing on MM medium is unclear. Nutrient utilisation during fungal growth was shown to be most efficient when carbon and nitrogen are present in a ratio of about 8:1 (Roels, 1983). In the studies undertaken here, SDA medium represented C:N ratio of approximately 20:1. This ratio is regarded as optimal in media used for production of *Colletotrichum truncatum* inocula with positive attributes such as high yield and assured quality (ie. high amounts of lipids and proteins) (Jackson & Schisler, 1992).

Although optimal for some pathogens, SDA medium may not be the best for continuous *in vitro* maintenance of these particular isolates since it contains high quantities of glucose. St Leger *et al.* (1994c) have demonstrated that *M. anisopliae* strains which germinated well and produced appressoria in glucose media were frequently related to the nutrient conditions pertaining to their hemipteran or lepidopteran hosts (insects

producing secretions rich in sugars), whereas strains isolated from coleopteran hosts were dGlc-resistant and germinated poorly on glucose. Since isolate V208 originates from an orthopteran host and isolates V245 and V234 originate from soil, it is likely that glucose, as an additional carbon source of SDA media, may be responsible for physiological changes observed in these isolates during continuous sub-culturing on glucose-containing SDA. It is well established that when provided with carbohydrates in excess of that required for balanced growth, many fungi such as *Aspergillus fischeri* (Prill *et al*, 1935), *C. truncatum* (Jackson & Schisler, 1992), *Beauveria bassiana*, *M. anisopliae*, *Paecilomyces fumosoroseus* (Hallsworth & Magan, 1994b), *Candida sake* (Teixidó *et al*, 1998b) and *C. coccodes* (Yu *et al,* 1998) transport and then convert these extra carbon sources into reserve carbohydrates such as lipids or polyols or excrete them as mucilageneous matrix. In media with high carbon concentrations and high C:N ratios (above 15:1) nitrogen becomes a growth-limiting factor. In this nitrogen-limited condition, *C. truncatum* and *C. coccodes* species are known to change their metabolic pathways and convert surplus raw materials into spore matrix (Jackson & Bothast, 1990; Yu *et al,* 1998). It would appear, therefore, that glucose as additional carbon source of SDA media (C:N of 20:1) used in this study, influenced the regulation and syntheses of endogenous compounds and surface properties of conidia produced.

Continuous sub-culturing of *Metarhizium* isolates on SDA media was shown to influence (increase or reduce) the ability of the fungus to assimilate some carbohydrates including glucose (Table 5.1). It could be suggested, therefore, that increased accumulation of endogenous reserves recorded for attenuated sub-cultures was a direct reflection of repeated sub-culturing influencing the absorption of glucose, which in turn influenced accumulation of reserve carbohydrates in conidia produced. Although there

was no relationship recorded in this study between individual polyols identified in conidia of isolates repeatedly sub-cultured on SDA and virulence as determined by LT_{50} values, a strong linear relationship ($r^2 = 0.50$; $P<0.001$) was established between total polyol content of the same conidia and virulence where increasing polyol content corresponded

to increased LT_{50} values, suggesting that increased amount of endogenous polyols would decrease the speed at which *M. anisopliae* was able to infect and kill its host.

Furthermore, the changes in glucose utilisation seemed to also affect the surface carbohydrates and surface proteins of fungal spores. For example, conidia of attenuated sub-cultures appeared to bind more calcofluor, a vital stain that binds to fungal surface 1-4-β-glucans (Butt *et al.* 1989; Ibrahim *et al.* 2002), than conidia of non-attenuated sub-cultures suggesting of increased expression of surface β-glucans on conidial surface of attenuated *M. anisopliae* sub-cultures. An increased expression of surface carbohydrate residues such as galactose-N-acetyl-galactosamine, α-D-mannose, N-acetyl-glucosamine, α-galactose, N-acetyl α-D-galactosaminyl and sialic acid, changes in molecular mass of α-D-mannosyl, α-D-glycosyl and α-(1-3) or (1-6) or (1-2) linked mannose carbohydrates (antigenic changes), structural and compositional changes occurring in carbohydrate moieties were all found to associate with continuous *in vitro* culture and subsequent attenuation of *C. salmositica* strains (Woo & Thomas, 1991; Feng & Woo, 2001). Moreover, when conidia of attenuated and non-attenuated sub-cultures of *M. anisopliae* isolates were subjected to formic (FA) or performic (PA) acid treatments, more FA-/PA-extractable surface proteins were obtained from attenuated sub-cultures than from those of non-attenuated (Plate 5.2a-b). However, when separated on SDS-PAGE gels these surface FA-soluble proteins appeared to have similar

molecular mass suggesting that no antigenic changes had occurred during continuous *in vitro* sub-culturing of *Metarhizium* isolates on SDA media. Nevertheless, when FA-soluble proteins were subsequently subjected to PA treatments and separated using SDS-PAGE, they all possessed more prominent bands of slightly higher molecular weight indicating that FA-soluble peptides from aerial conidia of all sub-cultures contained disulfide bonds, similar to those identified from surface rodlet layer of *B. bassiana* and *B.brongniartii* conidia (Bidochka *et al.* 1995; Jeffs *et al.* 1999).

It was previously demonstrated that hydrophobin complexes could be dissociated into their constituent monomers by treatment with FA or with oxidising agent such as performic acid (Wessels *et al.* 1991). The latter oxidises cysteine residues to cysteic acid, breaking disulphate bridges, and thereby relaxes the proteins and retards their migration through the polyacrylamide gel so that apparent molecular weight increases (Wessels, 1992, 1997). Since proteins extracted from conidia of attenuated and non-attenuated sub-cultures of *M. anisopliae* isolates resemble features found in fungal hydrophobins of *Schizophyllum commune* (de Vries *et* al. 1993), *B. bassiana* (Bidochka *et al.* 1995; Jeffs *et al.* 1999) and *B.brongniartii* (Jeffs *et al.* 1999) which are known to be low molecular weight proteins, SDS-insoluble but soluble in formic acid and when oxidised with performic acid the resultant proteins are of higher molecular weight, it would appear that they are indeed hydrophobin-like proteins. However, attenuated sub-cultures seemed to possess more total extractable hydrophobin-like surface protein than those of non-attenuated sub-cultures. One possible explanation to why surface hydrophobin-like proteins of attenuated sub-cultures are more readily removable than those of non-attenuated is that hydrophobin-like rodlet layers of attenuated sub-cultures may not be as tightly packed on the spore surfaces as those of non-attenuated sub-

cultures, thus facilitating their easy removal. Also, because of the loose structure of these hydrophobic layers, it may be that underlying carbohydrates become more accessible to and more recognisable by the calcofluor stain allowing more stain to be bound to the surface glucans resulting in increased spore fluorescence.

In conclusion, this study has shown that repeated *in vitro* sub-culturing of entomogenous fungus *M. anisopliae* induces physiological changes, such as the ability to assimilate sugars and their derivatives. This may in turn, influence the regulation, synthesis and accumulation of endogenous reserves, and affect the surface attributes of conidia, thus decreasing virulence. These changes are more prominent if fungal lines recycled through medium consisting, in excess, of readily available carbon such as glucose.

CHAPTER 6

General discussion

Entomopathogenic fungi (EPF) can provide safe and effective control of many important insect pests. Currently, economic interest lies in focusing upon fungi from three genera within the class *Hyphomycetes: Metarhizium, Beauveria* and *Verticillium.* There are numerous examples of the efficacious suppression of pest insects within this group of micro-organisms (Latge & Moletta, 1988; McCoy *et al.*, 1988; McCoy, 1990; Ferron *et al.*, 1991; Roberts & Hajek, 1992; Tanada & Kanya, 1993; Hajek & St Leger, 1994; Boucias & Pendland, 1998; Wraight & Carruthers, 1999; Butt & Copping, 2000; Lacey *et al.*, 2001; Butt *et al.*, 2001a) demonstrating their considerable potential as biological control agents (BCAs). However, their production and application has not always provided consistent control of insect pests. The factors responsible for such inconsistency are extremely complex, involving interactions among the pathogen(s), insect host, environment and time (Inglis, *et al.*, 2001). An understanding of these interactions and elucidation of the factors that limit disease initiation and development may allow us to overcome constraints and thereby achieve effective control of insect pests.

6.1 Nutrition and pathogenesis

Repeated *in vitro* culture has been shown to attenuate virulence of many fungal (Fargues & Roberts, 1983; Morrow *et al.*, 1989; Butt & Goettel, 2000; Canderone *et al.*, 2000; Singh *et al.*, 2001), bacterial (Kielian *et al.*, 2001), viral (Kiyotani *et al.*, 2001) pathogens and parasitic protozoa (Woo & Li, 1990; Feng & Woo, 2001). Methods to preserve pathogen virulence, including manipulation of media composition, have been explored for many years (Schaerffernberg, 1964; Fargues & Robert, 1983;

Lane *et al.*, 1991a; Yu *et al.*, 1998). Since optimisation of the production is critical to the successful commercial development of BCAs, medium optimisation schemes must be developed to improve propagule yield in conjunction with improvements in propagule 'fitness' for use as insecticides. Spore 'fitness' is equated with rapid germination, high rates of appressoria formation and enhanced tolerance to desiccation for increased product shelf-life (Wraight *et al.*, 2001).

Nutritional composition of the production media has been shown to influence germination, growth, conidiation, propagule yield, propagule attributes, desiccation tolerance, accumulation of endogenous reserve, virulence and biocontrol efficacy of fungal species (Fargues & Robert, 1983; Jackson & Bothast, 1990; Lane *et al.*, 1991a, 1991b; Schisler *et al.*, 1991; Silman *et al.*, 1993; Jenkins & Prior, 1993; Hallsworth & Magan, 1994a, 1994b, 1994c, 1995; Jackson *et al.*, 1997; Yu *et al.*, 1998).

Studies with submerged cultures of *Colletotrichum truncatum* led to an understanding of the nutritional regulation of conidia formation, yield and biocontrol efficacy (Jackson & Bothast, 1990; Schisler *et al.*, 1991). For example, when a carbon concentration of 4-16 g l^{-1} was used, high concentrations of conidia were produced. Carbon concentration greater than 25 g l^{-1}, however, was shown to inhibit conidiation and promoted the formation of highly melanized hyphal aggregates, known as microsclerotia (Jackson & Bothast, 1990). Media with a C:N ratio of 30:1consistently produced more conidia than media with C/N ratios of 10:1 or 80:1. However, conidia produced on a medium with a C:N ratio of 10:1 germinated more rapidly, formed appressoria more frequently and incited more disease in hemp (*Sesbania exaltata*) seedlings when compared with conidia produced in media with C:N ratios of 30:1 or

80:1 (Schisler *et al.*, 1991). Similarly, three isolates of *Metarhizium anisopliae* produced greater amount of conidia when grown on SDA medium than when grown on nutrient- poor minimal medium (MM) (Chapter 2). However, conidia of *M. anisopliae* derived from cultures grown on MM had higher germination potential when placed on the cuticles of *Myzus persicae* or *Meligethes aeneus* than conidia derived from nutrient rich SDA media (Chapter 2). A positive correlation between germination speed of conidia of various isolates of *Paecilomyces fumosoroseus* and infectivity on larvae of the diamondback moth, *Plutella xylostella* (Altre *et al.,* 1999) suggested that rapidly germinating spores have a significant advantage in causing infection under field condition, where unfavourable environment (fluctuating temperatures, limited free moisture, UV, etc.) represents a significant constraint on biocontrol efficacy.

Culture media has also been shown to cause changes in propagule attributes (Jackson & Bothast, 1990; Schisler *et al.*, 1991; Jackson & Schisler, 1992; Yu *et al,* 1998; Ibrahim *et al.*, 2002) which are important for determining virulence and pathogenicity. For example, conidia of *C. truncatum* produced in a medium with a C:N ratio of 10:1 were longer and thinner than those produced in 30:1 or 80:1 media (Schisler *et al.*, 1991). Unlike conidia of *M. anisopliae* grown on SDA or MM which were cylindrical (Chapter2, Plate 2.5b, d and k) and dark green in colour, conidia produced on SDA media amended with KCl had a distinct drop-like shape, coated in some deposits (Chapter 2, Plate 2.5h-i) and were unusually pale in colour. Excess carbohydrates in media with a C:N ratio higher than 15:1 are known to be excreted as mucilagenious spore matrixes (Jackson & Bothast, 1990; Yu *et al,* 1998). A strong linear relationship established between fluorescence intensity of calcofluor treated conidia and the number of conidia that adhered to the cuticle of potato-peach aphids where increasing spore

fluorescence corresponded to increased spore adhesion suggested that surface polysaccharides - β-glucans play an important role in either host recognition or spore adhesion for *M. anisopliae* (Chapter 2; Ibrahim *et al.*, 2002).

Surface glucans of bacterial *Rhizobium* (van der Drift *et al.*, 1998; Price, 1999; Spaink, 2000) and oral *Streptococcus* species (Doyle & Taylor, 1994) have also been shown to play an important role in the recognition of leguminous host plants and in determining the adhesion to teeth and gums, respectively. Culture media was also shown to significantly affect the surface carbohydrates such as α-D-mannose, α-D-glucose, β-D-galactose, N-acetyl-D-glucosamine of *M. anisopliae* conidia (Chapter 2; Ibrahim *et al.*, 2002). Since surface carbohydrates positively correlates to surface hydrophobicity (Jeffs *et al.*, 1999) it was suggested that production medium influences binding of spores to insect cuticles via hydrophobic forces by influencing the carbohydrates on conidial surfaces (Chapter 2; Ibrahim *et al.*, 2002). Furthermore, a strong relationship between LT_{50} values of isolate V245 and the number of conidia that successfully adhered to the surface of pollen beetle cuticle suggested that culture media influence the virulence of *M. anisopliae* by influencing the ability of conidia to adhere to the insect cuticles.

Since the quantitative fluorescence assay (Chapter 2) proved useful for the quantification of intensity of calcofluor fluorescence of stained conidia and showed a positive correlation between intensity of fluorescence and conidial adhesion to host cuticles, it can be concluded that adhesion of *M. anisopliae* conidia to insect cuticles is affected by the amount of β-glucans on spore surfaces. It could also be proposed that such a simple vital stain assay could prove useful in determining the potential virulence

of produced inoculum, whether it be for further laboratory studies or commercial production.

Different nutritional environments have been shown to influence the endogenous composite of conidia of *C. truncatum* (Jackson & Schisler, 1992), *B. bassiana, M. anisopliae* and *P. fumosoroseus* (Hallsworth & Magan, 1994a, 1994b), blastospores of *B. bassiana* (Lane *et al.,* 1991b) and yeast *Candida sake* (Teixidó *et al.*, 1998b). For example, conidia produced in media with a C:N ratio of 10:1 contained more protein and less lipid than conidia produced on media with a C:N ratio of 30:1 or 80:1 conidia (Jackson & Schisler, 1992). Producing bastospores of *B. bassiana* under nitrogen-limited conditions yielded inocula with higher amount of endogenous lipids and carbohydrates, including glycogen, than inocula from carbon-limited cultures (Lane *et al.,* 1991a). Furthermore, high concentrations of glycerol or trehalose in culture media significantly influenced the accumulation of endogenous polyhydroxy alcohols (polyols) and trehalose in conidia of *B. bassiana* and *M. anisopliae* produced (Hallsworth & Magan, 1994b). The results obtained from the study conducted in Chapter 2 showed that conidia of three isolates of *M. anisopliae* accumulated significantly higher concentrations of polyols and total proteins if grown on insect cuticles or nitrogen-limited medium (MM), when compared to conidia cultured on yeast extract agar (YEA) or SDA media. Moreover, conidia produced on SDA contained more glycerol but lacked trehalose, whilst conidia produced on MM contained no glycerol but accumulated higher quantities of arabitol and trehalose (Magan, 2001; Chapter 2). These results clearly demonstrate that the endogenous profiles of inocula produced on nutrient-rich media are significantly different from those in inocula obtained directly from insects or nutrient-poor media.

Several studies have suggested an association between increased efficacy of biocontrol, determined by an increased rate of germination, and increased endogenous protein, polyol or carbohydrate content (Lane *et al.,* 1991b; Jackson & Schisler, 1992; Hallsworth & Magan, 1994b). For example, media with a C:N ratio between 15:1 and 20:1 produced high concentration of *C. truncatum* conidia that were rich in protein, germinated rapidly, formed appressoria frequently and were highly efficacious in inciting disease in hemp sesbania seedlings. Conidia with higher concentrations of polyols and trehalose resulted in greater germination at lower water activity and caused more rapid infection and death of the larvae of *Galleria mellonella* at lower relative humidities (Hallsworth & Magan, 1994c). Blastospores of *B. bassiana* with higher lipid and carbohydrate content have also been shown to germinate and infect green leafhopper faster than conidia with lower amounts of endogenous reserves (Lane *et al.,* 1991a 1991b). Similarly, conida of *M. anisopliae* isolates cultured on MM were shown to germinate considerably faster, form appressoria more frequently, and incite faster infection and rapid kill of aphids and pollen beetles than conidia cultured on SDA (Chapter 2).

A direct relationship between endogenous nitrogen (N) and carbon (C) content and germination of conidia of three *M. anisopliae* isolates on insect cuticle suggests that these compounds play an important role in infection processes. Although there was no direct positive relationship found between individual polyol content and conidial germination in general, statistical analysis of the data presented in Chapter 2 suggested that increasing glycerol or mannitol content within conidial composition, significantly reduces the germination of *M. anisopliae.* Moreover, the results obtained from the study conducted in Chapter 2 also indicate that those culture media which encourage

the accumulation of high concentrations of mannitol affected conidial virulence, as measured by LT_{50} values, where increasing mannitol content corresponded to increased LT_{50}. A direct correlation between increased amount of erythritol and arabitol and decreased LT_{50} were found for conidia grown on different culture medium of two isolates *M. anisopliae*, suggesting that these polyols may be involved in fungal pathogenesis.

6.2 Nutrition and attenuation

Although an understanding of the effects of specific nutritional requirements of BCAs provides essential information for future successful commercialisation, the effect of prolonged or repeated culturing, *in vitro,* of BCA on reduced virulence, also known as attenuation, is critical for any industrial-scale production system. The most common solution in the avoidance of attenuation involves the storage of large quantities of inoculum taken either directly from insect hosts or from a limited number of *in vitro* passages (Jenkins *et al.,* 1998).

Prolonged or continued *in vitro* sub-culturing has been shown to change phenotype and/or morphology of many EPFs including *S. pracina, I. fumosorosea, O. destructor, A. flavus, A. oryzae, B. bassiana* (Kawakami, 1960), *V. lecanii* (Hall, 1980), *N. rileyi* (Lord & Roberts, 1986; Morrow *et al.,* 1989) and *E. neoaphidis* (Wilding *et al.,* 1992). In these studies, similar observations were recorded for three isolates of *M. anisopliae* (Chapter 3), when colony colour and morphology (increased sectoring and production of sterile colonies) were shown to change following three successive passages of the entomopathogen on SDA. However, following 11 consecutive passages of the pathogen

on MM media, no affect on the morphology or phenotype was observed for any of isolates tested. Hall (1980) demonstrated that 50 consecutive passages of *Verticillium lecanii* strain on SDA or Potato Dextrose agar (PDA) resulted in more than two morphological variants, whereas sub-culturing on Malt extract or Czapek-Dox media caused no morphological or phenotypic changes. These results clearly indicate that nutritional substrates significantly influence colonial morphology and phenotype, and effect the speed at which these changes take place during repeated sub-culturing.

Successive sub-culturing on artificial media was shown to have a significant effect on the production of sterile colonies (Wilding *et al.,* 1992, Chapter 3). However, sterile mycelium was shown (Chapter 3) to be encouraged to produce pathogenic conidia by simply damaging the hyphae. These observations, in addition to those recorded by Hall (1980), where conidia obtained from morphologically altered cultures of *V. lecanii* were shown to be as pathogenic as conidia obtained from parental cultures, suggests that factors affecting pathogen morphology may operate independently to those influencing pathogenicity.

Continuous sub-culturing of many micro-organisms is known to result in permanent (Hall, 1980; Woo & Li, 1990) or partial (Fargues & Roberts, 1983; Morrow *et al.*, 1989; Prenerova, 1994) attenuation of virulence. For example, reduced virulence of a *B. bassiana* isolate, as a result of successive sub-culturing (Kawakami, 1960), was restored by passaging the pathogen through silkworm larva. In contrast, an attenuated isolate of *V. lecanii* failed to increase virulence after passaging through the aphid, *Macrosiphoniella sanborni* (Hall, 1980). Recovery of virulence, assessed by determination of LT_{50}, was observed for *M. anisopliae* in the absence of passaging

through a host insect (Chapter 3). For example, successive sub-culturing of the pathogen on SDA medium, resulted in an increase in LT_{50} value following sub-culture 3 (V234), 5 (V245) or 11 (V208) depending on the isolate tested.

One possible explanation for such changes in LT_{50} values is the change in the physiological behaviour of fungal strains associated with *in vitro* sub-culturing. Indeed, repeated sub-culturing on both SDA and MM media of all three isolates of *M. anisopliae* was shown to affect the adhesion and germination of conidia both *in vitro* and *in vivo* (Chapter 3). For example, although the number of conidia that adhered to aphid cuticles increased with successive sub-culturing, conidia were slower to germinate and often failed to produce appressoria. As such, higher LT_{50} values were recorded. Conversely, for those few conidia of non-attenuated sub-cultures (inocula with low LT_{50} values) that successfully attached to insect cuticles, germination was faster and the proportion of germlings producing appressoria was higher, suggesting that virulent inocula required very few propagules to initiate rapid infection and high aphid mortality. In addition, virulent inocula consistently produced shorter germ-tubes and larger appressoria than less virulent inocula. However, these observations are in contrast to those recorded during *in vitro* studies, where although fewer conidia of attenuated isolate V245 (sub-culture 3) were shown to adhere to an artificial surface, the incidence of germination and appressorial development was observed to be high.

Discrepancies in the physiological behaviour of *M. anisopliae* sub-cultures may be due to the disruption of various pathways involved in determining morphogenesis and/or virulence. Repeated *in vitro* sub-culturing of *Candida albicans* has been shown to trigger the disruption of the N-acetylglucosamine (GlcNAc) catabolic pathway gene

cluster, thus causing reduction in pathogen adherence to human epithelial cells *in vitro,* and causing an inability to grow on amino sugars (Singh *et al.*, 2001). The API 50 CH test (Chapter 5) used to determine the effects of sub-culturing on carbon utilisation by *M. anisopliae,* suggested that continuous sub-culturing on SDA media influenced the ability of the pathogen to absorb various carbohydrates including glucose. This in turn influenced the accumulation of reserve carbohydrates such as glycerol and mannitol in conidia of *M. anisopliae,* and affected the expression of surface carbohydrates and surface proteins. The increased concentration of these polyols in conidial composition may be responsible for slow germination of attenuated conidia on aphid cuticles. Attenuated sub-cultures were also found to bind more calcofluor than conidia of non-attenuated sub-cultures suggesting an increased expression of surface β-glucans on the conidial surfaces of attenuated *M. anisopliae* sub-cultures. An increase in β-glucans has previously been proposed to explain the increased adherence of attenuated *M. anisopliae* conidia to aphid cuticles (Ibrahim *et al.,* 2002). In addition, increases in the expression of surface carbohydrate residues including galactose-N-acetyl-galactosamine, α-D-mannose, N-acetyl-glucosamine, α-galactose, N-acetyl α-D-galactosaminyl and sialic acid, changes in molecular mass of α-D-mannosyl, α-D-glycosyl and α-(1-3) or (1-6) or (1-2) linked mannose carbohydrates (antigenic changes), along with structural and compositional changes occurring in carbohydrate moieties, have also been suggested to explain the attenuation of *C. salmositica* strains associated with continuous *in vitro* sub-culturing (Woo & Thomas, 1991; Feng & Woo, 2001).

Attenuation of virulence of the parasitic protozoa *Cryptobia salmositica* (Woo & Thomas, 1991) and the entomopathogens *N. rileyi* (Ignoffo *et al.,* 1982) and *M.*

anisopliae (Chapter 3) may be associated with changes in protein profile. For example, an esterase profile for conidia of *M. anisopliae* (isolate V245), obtained from sub-cultures showing reduced virulence, revealed significant changes in protein profile. Although isoelectrofocusing techniques used in Chapter 4 showed no changes in banding profile of water-extracted proteins, significant quantitative changes were observed due to repeated sub-culturing. This indicates that repeated sub-culturing may produce multifarious biochemical alterations, which influence pathogen virulence and thus may interfere with the whole process of pathogenesis.

One of the major factors determining fungal virulence is the production of the correct viable enzymes in sufficient quantities and rate to facilitate rapid germination and penetration. Following successful attachment, conidia germinate and grow on the surface of an insect host. Penetration of the cuticle is usually preceded by the formation of an appressorium that firmly attaches the fungal propagule to the epicuticle and provides the fulcrum for mechanical and enzymatic processes which mediate penetration (Clarkson *et al.*, 1998). Since insect cuticle is biochemically a complex structure, EPFs require a number of hydrophilic enzymes in order to effect cuticular penetration and provide nutrients for growth. Subtilisin-like endoproteases Pr1a, Pr1b and Pr2 (St Leger *et al.*, 1992; Smithson *et al.*, 1995; Josh *et al.*, 1997), mettallo-endoproteases (St Leger *et al.*, 1994a), cysteine endoproteases (Cole *et al.*, 1993), metallo-aminopeptidases (St Leger *et al.*, 1993), serine dipeptidylpeptidases (St Leger *et al.*, 1993), carboxypeptidases (St Leger *et al.*, 1994b), chitinases (Valadares-Inglis *et al.*, 1997) and N-acetylglucosaminidases (St Leger *et al.*, 1991) have all been demonstrated to play an important role in the penetration of insect cuticle. Of these, the extracellular cuticle-degrading protease Pr1 has been studied the most intensely, having

shown to be the predominant protein produced during appressorium formation (St Leger *et al.*, 1987a, 1987b,1989). Given the complex of enzymes involved in cuticle degradation, the exact role of each individual enzyme is difficult to ascertain.

Quantitative enzyme assays, used to determine Pr1 activity for each sub-culture of *M. anisopliae* isolates (Chapter 4; Uribe, 1995) revealed that insect homogenate induced similar Pr1 production in all tested sub-cultures. This would, therefore, suggest that repeated sub-culturing of *M. anisopliae,* had no affect on Pr1 production. Similar size Pr1 products, as amplified by the nested PCR reaction, were also shown to be expressed by both non-attenuated and attenuated sub-cultures (Chapter 4), suggesting that continuous sub-culturing of the entomopathogen has no affect on *pr1* gene expression. Given that attenuated sub-cultures of *M. anisopliae* isolates (Chapter 4) resulted in lower aphid mortality, despite producing higher concentrations of Pr1, and that non-attenuated sub-cultures of the pathogen resulted in high aphid mortality, despite producing lower concentrations of Pr1, it would appear that Pr1 is a pathogenicity rather than virulence determinant. Furthermore, the induction/repression responses of *M. anisopliae* isolates were shown to be similar for all sub-cultures studied, indicating that regulatory controls at least for Pr1 protease are not affected by sub-culturing (Uribe, 1995). Studies, where attenuated strain of *B. bassiana* showed significantly lower protease activity in casein medium than in Sabouraud medium, suggested that the expression of gene products of entomopathogenic fungi that are required for enzymatic digestion of host proteins are regulated by nutrient levels rather than by prolonged culturing (Vilcinskas & Wedde, 1997). This suggestion is supported by findings reported in Chapter 4 where MM media supplemented with cockroach homogenate induced higher Pr1 activity than that of Sabouraud Dextrose broth.

Although pathogenicity of *M. anisopliae* isolates were not effected by continuous sub-culturing on both media (Chapter 4) and proved to be dose related, the key attributes of virulence were shown to be significantly affected, where increased LT_{50} and LC_{50} values corresponded with increased time and dose taken to initiate and complete disease development in aphid populations. Similar effects were observed by Ignoffo *et al.* (1982), who found that a 2-fold increase in conidial concentration of *Nomuraea rileyi* was required to kill 50% *Trichoplusia ni* larvae for, respectively, 12 and 18 serial passages on Sabouraud Maltose Agar. Hajek *et al.* (1990) also reported a two day increase in incubation time required for *Entomophaga maimaiga* protoplasts to cause death in a gypsy moth, *Lymantria dispar,* population following 15 serial sub-cultures.

6.3 Proposed further work

The results of this study have clearly shown that culture media influences the virulence of *M. anisopliae* by influencing the ability of conidia to adhere to and germinate on the cuticle of aphids and beetles. Furthermore, it has been demonstrated that adhesion of conidia to insect cuticle is affected by the amount of β-glucans on spore surfaces. It was also identified that not only does culture media influence the virulence of *M. anisopliae* isolates, but also that continuous sub-culturing on artificial media significantly affects the pathogen's physiological qualities (such as presence or absence of erythritol), arabitol and the balance of carbon and nitrogen (C:N ratios) in conidial composition. In order to further our understanding of factors which influence physiological changes of infective propagules occurring during *in vitro* sub-culturing, the following areas of research are recommended:

1. Further studies involving isolates of different fungal species from a wider range of geographic locations in order to determine any relatedness in the physiological changes due to the effects of *in vitro* sub-culturing.

2. Data from Chapters 2, 3 and 5 suggest that both nutrition and repeated sub-culturing of *M. anisopliae* cause defects in the conidial cell wall or conidial surface structure. Therefore, further studies are necessary to investigate:

    a. The effects of different concentrations of sugars used in common culture media on the expression of cell surface components such as surface polysaccharides (e.g. β-glucans), surface carbohydrates (e.g. α-D-mannose, α-D-glucose, β-D-galactose and N-acetyl-D-glucosamine) and surface proteins (e.g. hydrophobin);
    b. The changes in the cell surface receptor(s) that may occur during *in vitro* sub-culturing and how this may affect pathogen-host interaction mechanisms;
    c. Isolation and characterisation of hydrophobin structure and properties of rodlet layers from attenuated and non-attenuated conidia in order to determine the link between this surface protein and fungal virulence;
    d. The effects of *in vitro* sub-culturing on glycosylation/deglycosylation of surface proteins such as hydrophobins and how this may affect fungal adherence properties.

3. The results from Chapter 4 suggest that repeated *in vitro* sub-culturing has no effect on cuticle-degrading enzyme (Pr1) activity/expression. However, further studies are recommended in order to develop a quantitative PCR-based assay for monitoring *in vitro* and *in vivo pr1* gene expression and for determination of the effects of continuous sub-culturing on the quantities of the *pr1* gene expressed.

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